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(54) Title: IMPROVED METHODS FOR TRANSFORMING PHAFFIA STRAINS, TRANSFORMED PHAFFIA STRAINS SO OBTAINED AND RECOMBINANT DNA IN SAID METHODS

(57) Abstract

The present invention provides recombinant DNA comprising a transcription promoter and a downstream sequence to be expressed, in operable linkage therewith, wherein the transcription promoter comprises a region found upstream of the open reading frame of a highly expressed *Phaffia* gene, preferably a glycolytic pathway gene, more preferably the gene coding for Glyceraldehyde-3-Phosphate Dehydrogenase. Further preferred recombinant DNAs according to the invention contain promoters of ribosomal protein encoding genes, more preferably wherein the transcription promoter comprises a region found upstream of the open reading frame encoding a protein as represented by one of the amino acid sequences depicted in any one of SEQIDNOs: 24 to 50. According to a further aspect of the invention an isolated DNA sequence coding for an enzyme involved in the carotenoid biosynthetic pathway of *Phaffia rhodozyma* is provided, preferably wherein said enzyme has an activity selected from isopentenyl pyrophosphate isomerase activity, geranylgeranyl pyrophosphate synthase activity, phytoene synthase activity, phytoene desaturase activity and lycopene cyclase activity, still more preferably those coding for an enzyme having an amino acid sequence selected from the one represented by SEQIDNO: 13, SEQIDNO: 15, SEQIDNO: 17, SEQIDNO: 19, SEQIDNO: 21 or SEQIDNO: 23. Further embodiments concern vectors, transformed host organisms, methods for making proteins and/or carotenoids, such as astaxanthin, and methods for isolating highly expressed promoters from *Phaffia*.

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Improved methods for transforming *Phaffia* strains, transformed *Phaffia* strains so obtained and recombinant DNA in said methods

Technical field

The present invention relates to methods for transforming *Phaffia* yeast, transformed *Phaffia* strains, as well as recombinant DNA for use therein.

Background of the invention

Methods for transforming the yeast *Phaffia rhodozyma* have been disclosed in European patent application 0 590 707 A1. These methods involve incubation of protoplasts with DNA or incubation of *Phaffia* cells with DNA followed by lithium acetate treatment. The recombinant DNA used to transform *Phaffia* strains with either of these methods comprised a *Phaffia* actin gene promoter to drive expression of the selectable marker genes coding for resistance against G418 or phleomycin. The methods involve long PEG and lithium acetate incubation times and transformation frequencies are low. When protoplasts are used, the transformation frequency is dependent on the quality of the protoplast suspension, making the procedure less reliable.

Recently a method for transforming *Phaffia* strains has been reported by Adrio J.L. and Veiga M.(July 1995, Biotechnology Techniques Vol. 9, No. 7, pp. 509-512). With this method the transformation frequencies are in the range of 3 to 13 transformants per μ g DNA, which is low. A further disadvantage of the method disclosed by these authors consists in increased doubling time of the transformed cells. The authors hypothesised that this may be due to interference of the autonomously replicating vector with chromosome replication.

Clearly, there is still a need for a reliable and efficient method of transforming *Phaffia* strains with foreign DNA. It is an objective of the present invention to provide methods and means to achieve this. It is a further objective of the invention to optimize expression of certain genes in *Phaffia rhodozyma* in order to make *Phaffia* a more suitable production host for certain valuable compounds.

Summary of the invention

The invention provides a method for obtaining a transformed *Phaffia* strain, comprising the steps of contacting cells or protoplasts of a *Phaffia* strain with recombinant DNA under conditions conducive to uptake thereof, said recombinant DNA comprising a transcription promoter and a downstream sequence to be expressed which is heterologous to said transcription promoter, in operable linkage therewith, identifying *Phaffia rhodozyma* cells or protoplasts having obtained the said recombinant DNA in expressible form, wherein the transcription promoter comprises a region that is found upstream of the open reading frame of a highly expressed *Phaffia* gene. According to a preferred embodiment of the invention said highly expressed *Phaffia* gene is a glycolytic pathway gene, more preferably the glycolytic pathway gene is coding for Glyceraldehyde-3-Phosphate Dehydrogenase

(GAPDH). According to one aspect of the invention, said heterologous downstream sequence comprises an open reading frame coding for resistance against a selective agent, such as G418 or phleomycin.

Another preferred method according to the invention is one, wherein said recombinant DNA comprises further a transcription terminator downstream from the said DNA to be expressed, in operable linkage therewith, which transcription terminator comprises a region found downstream of the open reading frame of a *Phaffia* gene. It is still further preferred, that the recombinant DNA is in the form of linear DNA.

Another preferred embodiment comprises, in addition to the steps above, the step of providing an electropulse after contacting of *Phaffia* cells or protoplasts with DNA.

According to another embodiment the invention provides a transformed *Phaffia* strain capable of high-level expression of a heterologous DNA sequence, which strain is obtainable by a method according to the invention. Preferably, said *Phaffia* strain contains at least 10 copies of the said recombinant DNA integrated into its genome, such as a chromosome, particularly in the ribosomal DNA locus of said chromosome.

The invention also provides recombinant DNA comprising a transcription promoter and a heterologous downstream sequence to be expressed, in operable linkage therewith, wherein the transcription promoter comprises a region found upstream of the open reading frame of a highly expressed *Phaffia* gene, preferably a glycolytic pathway gene, more preferably a gene coding for Glyceraldehyde-3-Phosphate Dehydrogenase.

Also provided is recombinant DNA according to the invention, wherein the heterologous downstream sequence comprises an open reading frame coding for reduced sensitivity against a selective agent, preferably G418 or phleomycin. Said recombinant DNA preferably comprises further a transcription terminator downstream from the said heterologous DNA sequence to be expressed, in operable linkage therewith.

Further aspects of the invention concern a microorganism harbouring recombinant DNA according to the invention, preferably *Phaffia* strains, more preferably *Phaffia rhodozyma* strains, as well as cultures thereof.

According to still other preferred embodiments isolated DNA fragments are provided comprising a *Phaffia* GAPDH-gene, or a fragment thereof, as well as the use of such a fragment for making a recombinant DNA construct. According to one embodiment of this aspect said fragment is a regulatory region located upstream or downstream of the open reading frame coding for GAPDH, and it is used in conjunction with a heterologous sequence to be expressed under the control thereof.

The invention according to yet another aspect, provides a method for producing a protein or a pigment by culturing a *Phaffia* strain under conditions conducive to the production of said protein or pigment, wherein the *Phaffia* strain is a transformed *Phaffia* strain according to the invention.

According to another aspect of the invention, a method for obtaining a transformed *Phaffia* strain, comprising the steps of

contacting cells or protoplasts of a *Phaffia* strain with recombinant DNA under conditions conducive to uptake thereof,

said recombinant DNA comprising a transcription promoter and a downstream sequence to be expressed in operable linkage therewith,

identifying *Phaffia rhodozyma* cells or protoplasts having obtained the said recombinant DNA in expressible form,

wherein the downstream sequence to be expressed comprises an isolated DNA sequence coding for an enzyme involved in the carotenoid biosynthetic pathway of *Phaffia rhodozyma*. Preferably, said enzyme has an activity selected from geranylgeranyl pyrophosphate synthase (*crtE*), phytoene synthase (*crtB*), phytoene desaturase (*crtI*) and lycopene cyclase (*crtY*), more preferably an enzyme having an amino acid sequence selected from the one represented by SEQIDNO: 13, SEQIDNO: 15, SEQIDNO: 17 and SEQIDNO: 19. According to a further embodiment, the transcription promoter is heterologous to said isolated DNA sequence, such as a glycolytic pathway gene in *Phaffia*. Especially preferred according to this embodiment is the Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) gene promoter.

Also provided is a transformed *Phaffia* strain obtainable by a method according to the invention and capable of expressing, preferably over-expressing the DNA sequence encoding an enzyme involved in the carotenoid biosynthesis pathway gene.

The invention is also embodied in recombinant DNA comprising an isolated DNA sequence according to the invention, preferably in the form of a vector.

Also claimed is the use of such a vector to transform a host, such as a *Phaffia* strain.

A host obtainable by transformation, optionally of an ancestor, using a method according to any one of claims 1 to 5, wherein said host is preferably capable of over-expressing DNA according to the invention.

According to a further embodiment a method is provided for expressing an enzyme involved in the carotenoid biosynthesis pathway, by culturing a host according to the invention under conditions conducive to the production of said enzyme. Also provided is a method for producing a carotenoid by cultivating a host according to the invention under conditions conducive to the production of carotenoid.

The following figures further illustrate the invention.

Description of the Figures

Fig. 1. Mapping of the restriction sites around the *Phaffia rhodozyma* GAPDH gene. Ethidium bromide stained 0.8 % agarose gel (A) and Southern blot of chromosomal DNA (B) and cosmid pPRGDHcos1 (C) digested with several restriction enzymes and hybridized with the 300-bp PCR fragment of the *Phaffia rhodozyma* GAPDH gene. Lane 1, DNA x *Kpn*I; 2, x*Pst*I; 3, x*Sma*I; 4, x*Sph*I; L, lambda DNA digested with *Bst*EII; 5, x*Sst*I; 6, x*Xba*I and 7, x*Xho*I.

The blot was hybridized in 6 x SSC, 5 x Denhardt's, 0.1 % SDS, 100 ng/ml herring sperm DNA at 65°C and washed with 0.1 x SSC/0.1% SDS at 65°C. Exposure time of the autoradiogram was 16 h for the cosmid and 48 h from the blot containing the chromosomal DNA.

Fig. 2. The organisation of two subclones; pPRGDH3 and derivative (A) and pPRGDH6 and derivatives (B) containing (a part of) the GAPDH gene of *Phaffia rhodozyma*. The PCR probe is indicated by a solid box. The direction and extent of the sequence determination is indicated by arrows.

solid boxes: GAPDH coding sequence

open box: 5' upstream and promoter region of GAPDH

open box: 3' non-coding *Phaffia rhodozyma* GAPDH sequence

solid line: GAPDH intron

hatched box: Poly-linker containing sites for different restriction enzymes

dotted line: deleted fragments

Fig. 3. Cloning diagram of *Phaffia* transformation vector; pPR2.

solid box: 5' upstream and promoter sequence of GAPDH

hatched box: G418

solid line: pUC19

open box: ribosomal DNA of *Phaffia rhodozyma*

Only restriction sites used for cloning are indicated.

Fig. 4. Construction of pPR2T from pPR2T.

Solid box (*Bam*HI - *Hind*III fragment): GAPDH transcription terminator from *Phaffia*.

All other boxes and lines are as in Fig. 3. Only relevant details have been depicted.

Fig. 5. Detailed physical map of pGB-Ph9. bps = basepairs; rDNA ribosomal DNA locus of *Phaffia*; act.pro 2 = actin transcription promoter; act.1 5' non-translated and aminoterminal region of the open reading frame; NON COD. = non-coding region downstream of G418-gene;

Fig. 6. Detailed physical map of pPR2. GPDHpro = GAPDH transcription promoter region from *Phaffia*. Other acronyms as in Fig. 5.

Fig. 7. Detailed physical map of pPR2T. Tgdh = GAPDH transcription terminator of *Phaffia*. All other acronyms as in Fig. 5 and 6.

Fig. 8. Overview of the carotenoid biosynthetic pathway of *Erwinia uredovora*.

Fig. 9. Representation of cDNA fragments and a restriction enzyme map of the plasmids pPRcrtE (A); pPRcrtB (B), pPRcrtL (C) and pPRcrtY (B).

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Detailed description of the invention

The invention provides in generalised terms a method for obtaining a transformed *Phaffia* strain, comprising the steps of

35 contacting cells or protoplasts of a *Phaffia* strain with recombinant DNA under conditions conducive to uptake thereof,

said recombinant DNA comprising a transcription promoter and a downstream sequence to be expressed which is heterologous to said transcription promoter, in operable linkage therewith,

identifying *Phaffia rhodozyma* cells or protoplasts having obtained the said recombinant DNA in expressible form,

wherein the transcription promoter comprises a region that is found upstream of the open reading frame of a highly expressed *Phaffia* gene.

In order to illustrate the various ways of practicing the invention, some embodiments will be highlighted and the meaning or scope of certain phrases will be elucidated.

5 The meaning of the expression recombinant DNA is well known in the art of genetic modification, meaning that a DNA molecule is provided, single or double stranded, either linear or circular, nicked or otherwise, characterised by the joining of at least two fragments of different origin. Such joining is usually, but not necessarily done *in vitro*. Thus, within the ambit of the claim are molecules which comprise DNA from different organisms or different genes of the same organism, or 10 even different regions of the same gene, provided the regions are not adjacent in nature. The recombinant DNA according to the invention is characterised by a transcription promoter found upstream of an open reading frame of a highly expressed *Phaffia* gene, fused to a heterologous DNA sequence. With heterologous is meant 'not naturally adjacent'. Thus the heterologous DNA sequence may be from a different organisms, a different gene from the same organism, or even of the same gene as the 15 promoter, provided that the downstream sequence has been modified, usually *in vitro*. Such modification may be an insertion, deletion or substitution, affecting the encoded protein and/or its entrance into the secretory pathway, and/or its post-translational processing, and/or its codon usage.

The strong transcription promoter according to the invention must be in operable linkage with the heterologous downstream sequence in order to allow the transcriptional and translational machinery 20 to recognise the starting signals. The regions upstream of open reading frames of highly expressed *Phaffia* genes contain TATA-like structures which are positioned at 26 to about 40 nucleotides upstream of the cap-site; the latter roughly corresponds with the transcriptional start site. Thus in order to allow transcription of the heterologous downstream sequence to start at the right location similar distances are to be respected. It is common knowledge, however, that there is a certain tolerance in the location of the 25 TATA-signal relative to the transcription start site. Typically, mRNAs of the eukaryotic type contain a 5'-untranslated leader sequence (5'-utl), which is the region spanning the transcription start site to the start of translation; this region may vary from 30 to more than 200 nucleotides. Neither the length nor the origin of the 5'-utl is very critical; preferably it will be between 30 and 200 nucleotides. It may be from the same gene as the promoter, or it may be from the gene coding for the heterologous protein. It 30 is well known that eukaryotic genes contain signals for the termination of transcription and/or polyadenylation, downstream of the open reading frame. The location of the termination signal is variable, but will typically be between 10 and 200 nucleotides downstream from the translational stop site (the end of the open reading frame), more usually between 30 and 100 nucleotides downstream from the translational stop site. Although the choice of the transcription terminator is not critical, it is found, 35 that the when the terminator is selected from a region downstream of a *Phaffia* gene, preferably of a highly expressed *Phaffia* gene, more preferably from the GAPDH-encoding gene, the level of expression, as well as the frequency of transformation is improved.

It was found that significant numbers of clones were obtained which could grow on very high G418 concentrations (up to, and over, 1 mg/ml). Transcription promoters according to the invention are

said to be from highly expressed genes, when they can serve to allow growth of transformed *Phaffia* cells, when linked to a G418 resistance gene as disclosed in the Examples, in the presence of at least 200 µg/ml, preferably more than 400, even more preferably more than 600, still more preferably more than 800 µg/ml of G418 in the growth medium. Examples of such promoters are, in addition to the promoter upstream from the GAPDH-gene in *Phaffia*, the promoters from *Phaffia* genes which are homologous to highly expressed genes from other yeasts, such as *Pichia*, *Saccharomyces*, *Kluyveromyces*, or fungi, such as *Trichoderma*, *Aspergillus*, and the like. Promoters which fulfill the requirements according to the invention, may be isolated from genomic DNA using molecular biological techniques which are, as such, all available to the person skilled in the art. The present invention provides a novel strategy for isolating strong promoters from *Phaffia* as follows. A cDNA-library is made from *Phaffia* mRNA, using known methods. Then for a number of clones with a cDNA insert, the DNA fragment (which represents the cDNA complement of the expressed mRNA) is sequenced. As a rule all fragments represent expressed genes from *Phaffia*. Moreover, genes that are abundantly expressed (such as the glycolytic promoters) are overrepresented in the mRNA population. Thus, the number of DNA-fragments to be sequenced in order to find a highly expressed gene, is limited to less than 100, probably even less than 50. The sequencing as such is routine, and should not take more than a couple of weeks. The nucleotide sequences obtained from this limited number of fragments, is subsequently compared to the known sequences stored in electronic databases such as EMBL or Geneseq. If a fragment shows homology of more than 50% over a given length (preferably more than 100 basepairs) the fragment is likely to represent the *Phaffia* equivalent of the gene found in the electronic database. In yeasts other than *Phaffia*, a number of highly expressed genes have been identified. These genes include the glycolytic pathway genes, phosphoglucoisomerase, phosphofructokinase, phosphotrioseisomerase, phosphoglucomutase, enolase, pyruvate kinase, alcohol dehydrogenase genes (EP 120 551, EP 0 164 556; Rosenberg S. et al., 1990, *Meth. Enzymol.*: 185, 341-351; Tuite M.F. 1982, *EMBO J.* 1, 603-608; Price V. et al., 1990, *Meth. Enzymol.* 185, 308-318) and the galactose regulon (Johnston, S.A. et al., 1987, *Cell* 50, 143-146). Accordingly, those *Phaffia* cDNA fragments that are significantly homologous to the highly expressed yeast genes (more than 40%, preferably more than 50% identity in a best match comparison over a range of more than 50, preferably more than 100 nucleotides) should be used to screen a genomic library from *Phaffia*, to find the corresponding gene. Employing this method, 14 highly expressed mRNAs from *Phaffia rhodozyma* have been copied into DNA, sequenced, and their (putative) open reading frames compared to a nucleic acid and amino amino acid sequence databases. It turned out that 13 out of these fourteen cDNAs coded for ribosomal protein genes, of which one coded simultaneously to ubiquitin; one cDNA codes for a glucose-repressed gene. The isolation of the genes and the promoters usually found upstream of the coding regions of these genes is now underway, and it is anticipated that each of these transcription promoters may advantageously be used to express heterologous genes, such as carotenoid biosynthesis genes. Among the genes and transcription promoters especially preferred according to this invention are the promoter found upstream of the ubiquitin-ribosomal 40S protein corresponding to the cDNA represented in SEQIDNO:10, the glucose-repressed cDNA represented in SEQIDNO:26, the 40S ribosomal protein S27 encoding cDNA represented in

SEQIDNO:28, the 60S ribosomal protein P1 α encoding cDNA represented by SEQIDNO:30, the 60S ribosomal protein L37e encoding cDNA represented in SEQIDNO:32, the 60S ribosomal protein L27a encoding cDNA represented in SEQIDNO:34, the 60S ribosomal protein L25 encoding cDNA represented in SEQIDNO:36, the 60S ribosomal protein P2 encoding cDNA represented in SEQIDNO:38, the 40S ribosomal protein S17A/B encoding cDNA represented in SEQIDNO:40, the 40S ribosomal protein S31 encoding cDNA represented in SEQIDNO:42, the 40S ribosomal protein S10 encoding cDNA represented in SEQIDNO:44, the 60S ribosomal protein L37A encoding cDNA represented in SEQIDNO:46, the 60S ribosomal protein L34 encoding cDNA represented in SEQIDNO:48, or the 40S ribosomal protein S16 encoding cDNA represented in SEQIDNO:50.

Promoters from these or other highly expressed genes can be picked up by the method according to the invention using only routine skills of (a) making a cDNA library on mRNA isolated from a *Phaffia* strain grown under desired conditions, (b) determining (part of) the nucleotide sequence of the (partial) cDNAs obtained in step (a), (c) comparing the obtained sequence data in step (b) to known sequence data, such as that stored in electronic databases, (d) cloning putative promoter fragments of the gene located either directly upstream of the open reading frame or directly upstream of the transcription start site of the gene corresponding to the expressed cDNA, and (e) verifying whether promoter sequences have been obtained by expressing a suitable marker, such as the G418 resistance gene, or a suitable non-selectable "reporter" sequence downstream from a fragment obtained in (d), transforming the DNA into a *Phaffia rhodozyma* strain and determining the level of expression of the marker gene or reporter sequence of transformants. A transcriptional promoter is said to be of a highly expressed gene if it is capable of making *Phaffia rhodozyma* cells transformed with a DNA construct comprising the said promoter linked upstream of the G418 resistance marker resistant to G418 in concentrations exceeding 200 μ g per liter culture medium, preferably at least 400, more preferably more than 600 μ g/l. Especially preferred promoters are those conferring resistance against more than 800 μ g/ml G418 in the growth medium.

Optionally, the transcriptional start site may be determined of the gene corresponding to the cDNA corresponding to a highly expressed gene, prior to cloning the putative promoter sequences; this may serve to locate the transcriptional initiation site more precisely, and moreover, helps to determine the length of the 5'-non-translated leader of the gene. To determine the location of the transcription start site, reverse primer extension, or classical S1-mapping may be performed, based on the knowledge of the cDNA sequence. Thus the exact location of the transcription promoter can be determined without undue burden, and the isolation of a fragment upstream of the transcription start site and containing the promoter, from a hybridising genomic clone (for example a phage or cosmid) is routine. Cloning the putative promoter fragment in front (upstream) of the coding region of, for example the G418-resistance gene, and transforming the gene cassette to *Phaffia* in order to evaluate the level of G418 resistance, and hence the level of expression of the G418-resistance gene as a consequence of the presence of the promoter is routine.

In a manner essentially as described for the isolation of other strong promoters, above, a transcription terminator may be isolated, with the proviso, that the terminator is located downstream

from the open reading frame. The transcription stop site can be determined using procedures which are essentially the same as for the determination of the transcription start site. All these procedures are well known to those of skill in the art. A useful handbook is Nucleic Acid Hybridisation, Edited by B.D. Hames & S.J. Higgins, IRL Press Ltd., 1985; or Sambrook, *sub.* However, it is not critical that the transcription terminator is isolated from a highly expressed *Phaffia* gene, as long as it is from an expressed gene.

Using recombinant DNA according to the invention wherein the open reading frame codes for reduced sensitivity against G418, a transformation frequency was obtained up to 160 transformants per μ g of linear DNA, at a G418 concentration in the medium of 40 μ g/ml.

About 10 to 20 times as much transformed colonies were obtained with the vector according to the invention (pPR2) than with the prior art vector pGB-Ph9, disclosed in EP 0 590 707 A1 (see Table 2; in the experiment of Example 7, the improvement is even more striking).

The method according to the invention calls for conditions conducive to uptake of the recombinant DNA. Such conditions have been disclosed in EP 509 707. They include but are not limited to the preparation of protoplasts using standard procedures known to those of skill in the art, and subsequent incubation with the recombinant DNA. Alternatively, *Phaffia* cells may be incubated overnight in the presence of LiAc and recombinant DNA. Still further alternative methods involve the use of particle acceleration. According to a preferred embodiment, the conditions conducive to uptake involve electroporation of recombinant DNA into *Phaffia* cells, such as described by Faber et al., (1994, Current Genetics 25, 305-310). Especially preferred conditions comprise electroporation, wherein the recombinant DNA comprises *Phaffia* ribosomal DNA, said recombinant DNA being in the linear form, most preferably by cleaving said recombinant DNA in the said ribosomal region. Still further preferred conditions, comprise the use of recombinant DNA in amounts of between 1 and 10 μ g per 10^8 cells, more preferably about 5 μ g recombinant DNA is used per 2×10^8 cells,

which are cultivated for 16 h at 21°C.

Once cells have been transformed according to the method, identification of transformed cells may take place using any suitable technique. Thus, identification may be done by hybridisation techniques, DNA amplification techniques such a polymerase chain reaction using primers based on the recombinant DNA used, and the like. A preferred method of identifying transformed cells is one which employs selection for the recombinant DNA that comprises a gene coding for reduced sensitivity against a selective agent. A useful selective agent is G418, hygromycin, phleomycin and *amdS*. Genes that code for reduced sensitivity against these selective agents are well known in the art. The open reading frames of these genes may be used as the heterologous downstream sequence according to the invention, allowing selective enrichment of transformed cells, prior to identification of transformed cells. Once transformed cells have been identified they may used for further manipulation, or used directly in the production of valuable compounds, preferably in large scale fermentors.

It will be clear, that a very efficient method for transforming *Phaffia* strains has been disclosed. Moreover, not only the frequency of transformation is high, the expression levels of the transforming DNA is very high as well, as is illustrated by the exceptionally high resistance against

G418 of the transformed *Phaffia* cells when the open reading frame of the G418-resistance gene was fused to a promoter according to the invention when compared to the G418 resistance gene under control of the actin promoter in pGB-Ph9. It is concluded, therefore, that the GAPDH-promoter is a high-level transcriptional promoter that can be suitably used in conjunction with any heterologous DNA sequence, in order to reach high expression levels thereof in *Phaffia* strains.

It will be clear that the availability of new expression tools, in the form of the recombinant DNA according to the invention, creates a wealth of possibilities for producing new and valuable biomolecules in *Phaffia*.

Preferably, the downstream sequence comprises an open reading frame coding for proteins of interest. For example genes already present in *Phaffia*, such as those involved in the carotenoid pathway, may be manipulated by cloning them under control of the high-level promoters according to the invention. Increased expression may change the accumulation of intermediates and/or end-products or change the pathway of β-carotene, canthaxanthin, astaxanthin and the like. The overexpression of the crtB gene from *Erwinia uredovora* will likely increase astaxanthin levels, as the product of this gene is involved in the rate limiting step. The expression of a protein of interest may also give rise to xanthophylls not known to be naturally produced in *Phaffia*, such as zeaxanthin. An open reading frame that may be suitably employed in such a method includes but is not limited to the one encoding the protein producing zeaxanthin (crtZ gene) obtained from *Erwinia uredovora* (Misawa et al. 1990. J.Bacteriol. 172 : 6704-6712). Other carotenoid synthesis genes can be obtained for example from *Flavobacterium* (a gram-positive bacterium), *Synechococcus* (a cyanobacterium) or *Chlamydomonas* or *Dunaliella* (algae). Obviously, carotenoid synthesis genes of a *Phaffia* strain, once the genes have been isolated and cloned, are suitably cloned into a recombinant DNA according to the invention and used to modify the carotenoid content of *Phaffia* strains. Examples of cloned carotenoid genes that can suitably be overexpressed in *Phaffia*, are those mentioned in Fig. 8. Particularly useful is crtE from *Phycomyces blakesleanus*, encoding Geranylgeranyl Diphosphate Synthase, and crtB, encoding phytoene synthase, as this step appears to be the rate-limiting step in carotenoid synthesis in *Thermus thermophylus* (Hoshino T. et al., 1994, Journal of Fermentation and Bioengineering 77, No. 4, 423-424). Especially preferred sources to isolate carotenoid biosynthetic genes or cDNAs from are the fungi *Neurospora crassa*, *Blakeslea trispora*. Other yeasts shown to possess cross-hybridizing species of carotenoid biosynthetic genes are *Cystofylobasidium*, e.g. *bisporidii* and *capitatum*.

Carotenoid biosynthesis genes have also been identified in plants; these plant cDNAs or genes from plants may be used as well. Optionally, the codon usage of the *Phaffia* genes or cDNAs may be adapted to the preferred use in the host organism.

Of special interest according to the present invention, are the DNA sequences coding for four different enzymes in the carotenoid biosynthesis pathway of *Phaffia rhodozyma*, represented in the sequence listing. It will be clear to those having ordinary skill in the art, that once these DNA sequences have been made available it will be possible to bring about slight modifications to the DNA sequence without modifying the amino acid sequence. Such modifications are possible due to the degeneracy of the genetic code. Such modifications are encompassed in the present invention. However, also

modifications in the coding sequences are envisaged that create modifications in the amino acid sequence of the enzyme. It is well known to those of skill in the art that minor modifications are perfectly permissible in terms of enzymatic activity. Most changes, such as deletions, additions or amino acid substitutions do not affect enzymatic activity, at least not dramatically. Such variants as comprise one or more amino acid deletions, additions or substitutions can readily be tested using the complementation test disclosed in the specification. The skilled person is also familiar with the term "conservative amino acid substitutions", meaning substitutions of amino acids by similar amino acids residing in the same group. The skilled person is also familiar with the term "allelic variant", meaning naturally occurring variants of one particular enzyme. These conservative substitutions and allelic enzyme variants do not depart from the invention.

As stated, at the DNA level considerable variation is acceptable. Although the invention discloses four DNA sequences, as represented in SEQIDNO: 12, SEQIDNO: 14, SEQIDNO: 16, SEQIDNO: 18, SEQIDNO: 20, or SEQIDNO: 22, in detail also isocoding variants of the DNA sequence represented in SEQIDNO: 12, SEQIDNO: 14, SEQIDNO: 16, SEQIDNO: 18, SEQIDNO: 20, or SEQIDNO: 22, are encompassed by the present invention. Those of skill in the art would have no difficulty in adapting the nucleic acid sequence in order to optimize codon usage in a host other than *P. rhodozyma*. Those of skill in the art would know how to isolate allelic variants of a DNA sequence as represented in SEQIDNO: 12, SEQIDNO: 14, SEQIDNO: 16, SEQIDNO: 18, SEQIDNO: 20, or SEQIDNO: 22 from related *Phaffia* strains. Such allelic variants clearly do not deviate from the present invention.

Furthermore, using the DNA sequences disclosed in the sequence listing, notably SEQIDNO: 12, SEQIDNO: 14, SEQIDNO: 16 or SEQIDNO: 18, as a probe, it will be possible to isolate corresponding genes from other strains, or other microbial species, or even more remote eukaryotic species if desired, provided that there is enough sequence homology, to detect the same using hybridisation or amplification techniques known in the art.

Typically, procedures to obtain similar DNA fragments involve the screening of bacteria or bacteriophage plaques transformed with recombinant plasmids containing DNA fragments from an organism known or expected to produce enzymes according to the invention. After *in situ* replication of the DNA, the DNA is released from the cells or plaques, and immobilised onto filters (generally nitrocellulose). The filters may then be screened for complementary DNA fragments using a labeled nucleic acid probe based on any of the sequences represented in the sequence listing. Dependent on whether or not the organism to be screened for is distantly or closely related, the hybridisation and washing conditions should be adapted in order to pick up true positives and reduce the amount of false positives. A typical procedure for the hybridisation of filter-immobilised DNA is described in Chapter 5, Table 3, pp. 120 and 121 in: *Nucleic acid hybridisation- a practical approach*, B.D. Hames & S.J. Higgins Eds., 1985, IRL Press, Oxford). Although the optimal conditions are usually determined empirically, a few useful rules of thumb can be given for closely and less closely related sequences.

In order to identify DNA fragments very closely related to the probe, the hybridisation is performed as described in Table 3 of Hames & Higgins, *supra*, (the essentials of which are reproduced

below) with a final washing step at high stringency in 0.1 * SET buffer (20 times SET = 3M NaCl, 20 mM EDTA, 0.4 M Tris-HCl, pH 7.8), 0.1% SDS at 68° Celsius).

To identify sequences with limited homology to the probe the procedure to be followed is as in Table 3 of Hames & Higgins, *supra*, but with reduced temperature of hybridisation and washing. A final wash at 2 * SET buffer, 50°C for example should allow the identification of sequences having about 75% homology. As is well known to the person having ordinary skill in the art, the exact relationship between homology and hybridisation conditions depend on the length of the probe, the base composition (% of G + C) and the distribution of the mismatches; a random distribution has a stronger decreasing effect on T_m than a non-random or clustered pattern of mismatches.

The essentials of the procedure described in Table 3, Chapter 5 of Hames & Higgins are as follows:

(1) prehybridisation of the filters in the absence of probe, (2) hybridisation at a temperature between 50 and 68°C in between 0.1 and 4 * SET buffer (depending on the stringency), 10 * Denhardt's solution (100 * Denhardt's solution contains 2% bovine serum albumin, 2% Ficoll, 2% polyvinylpyrrolidone), 0.1% SDS, 0.1% sodiumpyrophosphate, 50 µg/ml salmon sperm DNA (from a stock obtainable by dissolving 1 mg/ml of salmon sperm DNA, sonicated to a length of 200 to 500 bp, allowed to stand in a water bath for 20 min., and diluted with water to a final concentration of 1 mg/ml); hybridisation time is not too critical and may be anywhere between 1 and 24 hours, preferably about 16 hours (o/n); the probe is typically labeled by nick-translation using ^{32}P as radioactive label to a specific activity of between 5 * 10^7 and 5 * 10^8 c.p.m./µg; (3) (repeated) washing of the filter with 3 * SET, 0.1% SDS, 0.1% sodiumpyrophosphate at 68°C at a temperature between 50°C and 68°C (dependent on the stringency desired), repeated washing while lowering the SET concentration to 0.1%, wash once for 20 min. in 4 * SET at room temperature, drying filters on 3MM paper, exposure of filters to X-ray film in a cassette at -70°C for between 1 hour and 96 hours, and developing the film.

Generally, volumina of prehybridisation and hybridisation mixes should be kept at a minimum. All "wet" steps may be carried out in little sealed bags in a pre-heated water bath.

The above procedure serves to define the DNA fragments said to hybridise according to the invention. Obviously, numerous modifications may be made to the procedure to identify and isolate DNA fragments according to the invention. It is to be understood, that the DNA fragments so obtained fall under the terms of the claims whenever they can be detected following the above procedure, irrespective of whether they have actually been identified and/or isolated using this procedure.

Numerous protocols, which can suitably be used to identify and isolate DNA fragments according to the invention, have been described in the literature and in handbooks, including the quoted Hames & Higgins, *supra*).

With the advent of new DNA amplification techniques, such as direct or inverted PCR, it is also possible to clone DNA fragments *in vitro* once sequences of the coding region are known.

Also encompassed by the claims is a DNA sequence capable, when bound to nitrocellulose filter and after incubation under hybridising conditions and subsequent washing, of specifically hybridising to a radio-labelled DNA fragment having the sequence represented in SEQIDNO: 12,

SEQIDNO: 14, SEQIDNO: 16 or SEQIDNO: 18, as detectable by autoradiography of the filter after incubation and washing, wherein said incubation under hybridising conditions and subsequent washing is performed by incubating the filter-bound DNA at a temperature of at least 50°C, preferably at least 55°C, more preferably at least 60°C in the presence of a solution of the said radio-labeled DNA in 0.3 M NaCl, 40 mM Tris-HCl, 2 mM EDTA, 0.1% SDS, pH 7.8 for at least one hour, whereafter the filter is washed at least twice for about 20 minutes in 0.3 M NaCl, 40 mM Tris-HCl, 2 mM EDTA, 0.1% SDS, pH 7.8, at a temperature of 50°C, preferably at least 55°C, more preferably at least 60°C, prior to autoradiography.

The heterologous DNA sequence according to the invention may comprise any open reading frame coding for valuable proteins or their precursors, like pharmaceutical proteins such as human serum albumin, IL-3, insulin, factor VIII, tPA, EPO, α -interferon, and the like, detergent enzymes, such as proteases and lipases and the like, cell wall degrading enzymes, such as xylanases, pectinases, cellulases, glucanases, polygalacturonases, and the like, and other enzymes which may be useful as additives for food or feed (e.g. chymosin, phytases, phospholipases, and the like). Such genes may be expressed for the purpose of recovering the protein in question prior to subsequent use, but sometimes this may not be necessary as the protein may be added to a product or process in an unpurified form, for example as a culture filtrate or encapsulated inside the *Phaffia* cells.

The yeast cells containing the carotenoids can be used as such or in dried form as additives to animal feed. Furthermore, the yeasts can be mixed with other compounds such as proteins, carbohydrates or oils.

Valuable substances, such as proteins or pigments produced by virtue of the recombinant DNA of the invention may be extracted. Carotenoids can also be isolated for example as described by Johnson et al. (Appl. Environm. Microbiol. 35: 1155-1159 (1978)).

Purified carotenoids can be used as colorants in food and/or feed. It is also possible to apply the carotenoids in cosmetics or in pharmaceutical compositions.

The heterologous downstream sequence may also comprise an open reading frame coding for reduced sensitivity against a selective agent. The open reading frame coding for an enzyme giving G418 resistance was used satisfactorily in the method according to the invention, but the invention is not limited to this selection marker. Other useful selection markers, such as the phleomycin resistance gene may be used, as disclosed in EP 590 707. Each of these genes is advantageously expressed under the control of a strong promoter according to the invention, such as the GAPDH-promoter.

The invention is now being illustrated in greater detail by the following non-limitative examples.

Experimental

Strains: *E. coli* DH5 α : supE44 lacU169 (80lacZM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1
E. coli LE392: supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1
P. rhodozyma CBS6938

Plasmids:

pUC19 (Gibco BRL)

pTZ19R

PUC-G418

pGB-Ph9 (Gist-brocades)

pMT6 (1987, Breter H.-J., Gene 53, 181-190))

5 **Media:** LB: 10 g/l bacto tryptone, 5 g/l yeast extract, 10 g/l NaCl. Plates; +20 g/l bacto agar. When appropriate 50 µg/ml ampicillin.

YePD: 10 g/l yeast extract, 20 g/l bacto peptone, 20 g/l glucose. Plates; +20 g/l bacto agar.

When appropriate 50 µg/ml Geneticin (G418).

10 **Methods:** All molecular cloning techniques were essentially carried out as described by Sambrook *et al.* in Molecular Cloning: a Laboratory Manual, 2nd Edition (1989; Cold Spring Harbor Laboratory Press).

Enzyme incubations were performed following instructions described by the manufacturer. These incubations include restriction enzyme digestion, dephosphorylation and ligation (Gibco BRL).

15 Isolation of chromosomal DNA from *Phaffia rhodozyma* as described in example 3 of patent Gist-brocades; EP 0 590 707 A1. Chromosomal DNA from *K. lactic* and *S.cerevisiae* was isolated as described by Cryer *et al.* (Methods in Cell Biology 12: 39, Prescott D.M. (ed.) Academic Press, New York).

20 Isolation of large (> 0.5-kb) DNA fragments from agarose was performed using the Geneclean II Kit whereas small (< 0.5-kb) and DNA fragments or fragments from PCR mixtures were isolated using Wizard™ DNA Clean-Up System (Promega).

Transformation of *E. coli* was performed according to the CaCl₂ method described by Sambrook *et al.* Packaging of cosmid ligations and transfection to *E. coli* LE392 was carried out using the Packagene Lambda DNA Packaging System (Promega), following the Promega protocols.

25 Isolation of plasmid DNA from *E. coli* was performed using the QIAGEN (Westburg B.V. NL).

Transformation of *Phaffia* CBS6938 was done according to the method for *H. polymorpha* described by Faber *et al.*, *supra*;

- Inoculate 30 ml of YePD with 1 CBS6938 colony
- Grow 1-2 days at 21°C, 300 rpm (pre-culture)
- Inoculate 200 ml of YePD with pre-culture to OD₆₀₀ = between 0 and 1 (if above 1 dilute with water)
- Grown o/n at 21°C, 300 rpm until OD₆₀₀ = 1.2 (dilute before measuring)
- Centrifuge at 5 min. 8000 rpm, room temperature. Remove supernatant thoroughly
- Resuspend pellet in 25 ml 50 mM KPi pH 7.0, 25 mM DTT (freshly made)

Transfer suspension to a fresh sterile 30 ml centrifuge tube and incubate for 15 min. at room temperature

- Centrifuge 5 min. at 8000 rpm 4°C, remove supernatant thoroughly
- Resuspend pellet in 25 ml of ice cold STM (270 mM sucrose, 10 mM Tris pH 7.5, 1 mM MgCl₂)
- Centrifuge 5 min. at 8000 rpm, 4°C
- Repeat washing step
- Resuspend cells in 0.5 ml of ice cold STM (3*10⁹ cells/ml). Keep on ice!

- Transfer 60 μ l of cell suspension to pre-cooled Eppendorf tubes containing 5 μ g transforming DNA (use precooled tips!), Keep on ice
- Transfer Cell/DNA mix to precooled electroporation cuvettes (top to bottom)
- Pulse: 1.5 kV, 400 Ω , 25 μ F
- 5 - Immediately add 0.5 ml of ice cold YePD. Transfer back to ep using a sterile Pasteur pipette
- Incubate 2.5 hrs at 21°C
- Plate 100 μ l onto YePD-plates containing 40 μ g/ml G418
- Incubate at 21°C until colonies appear.

Pulsed Field Electrophoresis was performed using a GENE Navigator + accessories 10 (Pharmacia). Conditions: 0.15 * TBE, 450 V, pulse time 0.5 s, 1.2% agarose, run time 2 h.

Polymerase Chain Reaction (PCR) experiments were performed in mixtures having the following composition:

- 5 ng of plasmid DNA or 1 μ g chromosomal DNA
- 0.5 μ g of oligo nucleotides (5 μ g degenerated oligo's in combination with chromosomal 15 DNA)
- 10 nm of each dNTP
- 2.5 μ m KCl
- 0.5 μ m Tris pH 8.0
- 0.1 μ m MgCl₂
- 20 - 0.5 μ g gelatin
- 1.3 U *Taq* polymerase (5 U in combination with chromosomal DNA)

H₂O was added to a total volume of 50 μ l

Reactions were carried out in an automated thermal cycler (Perkin-Elmer).

Conditions: 5 min. 95°C , followed by 25 repeated cycles; 2' 94°C, 2' 45°C 3' 72°C
25 Ending ; 10 min. 72°C.

Fusion PCR reactions were performed as described above, except that 2 DNA fragments with compatible ends were added as a template in equimolar amounts.

Oligo nucleotide sequences were as follows:

- 30 3005: CGGGATCCAA(A/G)CTNACNGGNATGGC (SEQIDNO: 1);
- 3006: CGGGATCC(A/G)TAICC(C/A/G)(C/T)A(T/C)TC(A/G)TT(A/G)TC(A/G)TACCA (SEQIDNO: 2);
- 4206: GCGTGACTTCTGGCCAGCCACGATAGC (SEQIDNO: 3);
- 35 5126: TTCAATCCACATGATGGTAAGAGTGTTAGAGA (SEQIDNO: 4);
- 5127: CTTACCACATGTGGATTGAACAAGATGGAT (SEQIDNO: 5);

5177: CCCAAGCTTCTCGAGGTACCTGGTGGGTGCATGTATGTAC (SEQIDNO: 6);

5137: CCAAGGCCTAAAACGGATCCCTCCAAACCC (SEQIDNO: 7);

5 5138: GCCAAGCTTCTCGAGCTTGATCAGATAAAGATAGAGAT (SEQIDNO: 8);

Example 1

G-418 resistance of *Phaffia* transformant G418-1

To determine the expression of the G418 resistance gene in pGB-Ph9, transformant G418-1 (EP 0 590 707 A1) was exposed to increasing concentrations of G418. Two dilutions of a G418-1 culture were plated onto YepD agar containing 0-1000 µg/ml G418 (Table I).

[G418] µg/ml	<i>Phaffia</i> G418-1 Dil.=10 ⁻⁴ (OD ₆₀₀ =7)	<i>Phaffia</i> G418-1 Dil.=10 ⁻⁵ (OD ₆₀₀ =7)	<i>Phaffia</i> (CBS6938) Dil.=0(OD ₆₀₀ =5)
0	>300	74	>300
200	>300	70	0
300	>300	61	0
400	212	13	0
500	10	2	0
600	0	0	0
700	0	0	0
800	0	0	0
900	0	0	0
1000	0	0	0

25

Table I. Survival of *Phaffia* transformant G418-1 on YepD agar medium containing increasing concentrations of G418.

At a concentration of 600 µg/ml G418 less than 1% of the plated cells survived. It can be concluded, that despite multicopy integration of pGB-Ph9, G418-1 shows a rather weak resistance to G418 (Scorer *et al.*, 1994, *Bio/Technology* 12, p. 181 *et seq.*, Jimenez and Davies, 1980, *Nature* 187 p. 869 *et seq.*), most probably due to a weak action of the *Phaffia* actin promoter in the plasmid. The results that the *Phaffia* actin promoter works poorly, prompted us to isolate promoter sequences of *Phaffia* with strong promoter activity.

Example 2Synthesis of specific probes of glycolytic genes from *Phaffia rhodozyma* by PCR

The polymerase chain reaction (PCR) technique was used in an attempt to synthesize a homologous probe of the genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK) and the triose phosphate isomerase (TPI) of *Phaffia rhodozyma*.

A set of degenerated oligonucleotides was designed based on the conserved regions in the GAPDH-gene (Michels *et al.*, 1986. EMBO J. 5: 1049-1056), PGK-gene (Osinga *et al.*, 1985. EMBO J. 4: 3811-3817) and the TPI-gene (Swinkels *et al.*, 1986. EMBO J. 5: 1291-1298).

All possible oligo combinations were used to synthesize a PCR-fragment with chromosomal DNA of *Phaffia rhodozyma* (strain CBS6938) as template. Chromosomal DNA of *Saccharomyces cerevisiae* and *Kluyveromyces lactis* as template was used to monitor the specificity of the amplification. The PCR was performed as described above, the PCR conditions were 1' 95 °C, 2' annealing temperature (T_a), in 5' from annealing temperature to 72 °C, 2' 72 °C, for 5 cycles followed by 1' 95 °C, 2' 55 °C and 2' 72 °C for 25 cycles and another elongation step for 10' 72 °C. Three different T_a were used 40 °C, 45 °C and 50 °C.

Under these conditions, only one primer combination produced a fragment of the expected size on chromosomal DNA of *Phaffia* as template. Using the oligo combination no: 3005 and 3006 and a T_a of 45 °C a 0.3-kb fragment was found. Specifically, the GAPDH oligonucleotides correspond with amino acids 241-246 and 331-338 of the published *S. cerevisiae* sequence. (It was concluded that to isolate the promoters corresponding to the PGK- and TPI-genes from *Phaffia*, either further optimization of the PCR-conditions is required, or homologous primers should be used. Another alternative method for isolating high level promoters is disclosed in the detailed description, *supra*).

The amplified fragment was purified from the PCR reaction and was digested with *Bam*HI and ligated into the dephosphorylated *Bam*HI site of pTZ19R. The ligation mixture was transformed to competent *E. coli* DH5α cells prepared by the CaCl₂-method and the cells were plated on LB-plates with 50 µg/ml Amp and 0.1 mM IPTG/50 µg/ml X-gal. Plasmid DNA was isolated from the white colonies. The pTZ19R clone with the right insert, called pPRGDH1, was subsequently used for sequence analysis of the insert.

The cloned sequence encoded for the carboxy terminal fragment of GAPDH of *Phaffia* as shown by comparison with the GAPDH-gene sequence of *S. cerevisiae* (Holland and Holland, 1979. J. of Biol. Chem. 254: 9839-9845).

Example 3Isolation of the GAPDH-gene of *Phaffia*

To obtain the complete GAPDH-gene including expression signals the 0.3-kb *Bam*HI fragment of pPRGDH1 was used to screen a cosmid library of *Phaffia*.

Preparation of the vector for cosmid cloning.

Vector preparation was simplified, because of the presence of a double cos-site in pMT6. pMT6 was digested to completion with blunt end cutter *Pvu*II to release the cos-sites. Digestion efficiency was checked by transformation to *E. coli* DH5 α and found to be >99%.

The *Pvu*II digested pMT6 was purified by phenol:chloroform extraction and ethanol precipitation and finally solved in 30 μ l TE at a concentration of 2 μ g/ μ l.

The vector was subsequently digested with cloning enzyme *Bam*HI and the vector arms were purified as described above ("Experimental").

Preparation of target DNA

Isolation of genomic DNA of *Phaffia* strain CBS6938 was performed as described in the part named "Experimental". The cosmid pMT6 containing inserts of 25-38-kb are most efficiently packaged. Therefore genomic DNA was subjected to partial digestion with the restriction enzyme *Sau*3A. Target DNA was incubated with different amounts of enzyme. Immediately after digestion the reactions were stopped by the extraction of DNA from the restriction mixture with phenol-chloroform. The DNA was precipitated by using the ethanol method and the pelleted DNA after centrifugation was dissolved in a small volume of TE. Contour clamped homogeneous electric field (CHEF) electrophoresis was used to estimate the concentration and size of the fragments (Dawkins, 1989, *J. of Chromatography* **492**, pp. 615-639).

Construction of genomic cosmid library.

Ligation of approximately 0.5 μ g of vector arm DNA and 0.5 μ g of target DNA was performed in a total volume of 10 μ l in the presence of 5 mM ATP (to prevent blunt end ligation). Packaging in phage heads and transfection to *E. coli* LE 392 as described in Experimental. The primary library consisted of 7582 transfecants with an average insert of 28-kb as determined by restriction analysis. The library represents 3.5 times the genome with a probability of the presence of all genes in the library of 0.97 as calculated according to Sambrook (*supra*). For library amplification the transfecants were pooled by resuspending in 8 ml LB-broth. Additional 4.8 ml glycerol was added. The transfecants mixture was divided into 16 samples of 800 μ l each and stored at -80 °C. This amplified library consisted of 2.9×10^9 transfecants.

30

Screening of the cosmid library.

A 100 μ l sample was taken from this library and further diluted (10^6) in LB-broth and 200 μ l was plated onto 10 LB-plates containing ampicillin. The plates were incubated overnight at 37 °C. Each plate contained 300-400 colonies and filters were prepared. These filters were screened with the GAPDH-probe using hybridization and washing conditions as described above ("Experimental"). After 16 hours exposure, 3 strong hybridization signals were found on the autoradiogram. Cosmid DNA isolated from these positive colonies was called pPRGDHcos1, pPRGDHcos2 and pPRGDHcos3.

Chromosomal DNA isolated from *Phaffia rhodozyma* strain CBS 6938 and cosmid pPRGDHcos1 was digested with several restriction enzymes. The DNA fragments were separated, blotted and hybridized as described before. The autoradiograph was exposed for different time periods at -80°C. The film showed DNA fragments of different length digested by different restriction enzymes which hybridize with the GAPDH-probe (Fig. 1).

Furthermore, from Southern analysis of the genomic DNA of *Phaffia* using the GAPDH fragment as probe, it was concluded that the GAPDH-encoding gene is present as a single copy gene in *Phaffia rhodozyma*, whereas in *Saccharomyces cerevisiae* GAPDH is encoded by three closely related but unlinked genes (Boucherie *et al.*, 1995. FEMS Microb. Letters 135:127-134).

Hybridizing fragments of pPRGDHcos1 for which a fragment of the same length in the chromosomal DNA digested with the same enzyme was found, were isolated from an agarose gel. The fragments were ligated into the corresponding sites in pUC19. The ligation mixtures were transformed to competent *E. coli* cells. The plasmids with a 3.3-kb *Sal*I insert and a 5.5-kb *Eco*RI insert were called pPRGDH3 and pPRGDH6, respectively. The restriction map of pPRGDH3 and pPRGDH6 is shown in Figure 2. Analysis of the sequence data of the insert in pPRGDH1 showed us that there was a *Hind*III site at the C-terminal part of the GAPDH-gene. From this data it was suggested that the insert in pPRGDH6 should contain the complete coding sequence of GAPDH including promoter and terminator sequences.

Example 4

Characterization of the GAPDH-gene

In order to carry out sequence analysis without the need to synthesize a number of specific sequence primers a number of deletion constructs of plasmids pPRGDH3 and pPRGDH6 were made using convenient restriction sites in or near the putative coding region of GAPDH gene.

The plasmids were digested and after incubation a sample of the restriction mixture was analyzed by gel electrophoresis to monitor complete digestion. After extraction with phenol-chloroform the DNA was precipitated by ethanol. After incubation at -20 °C for 30' the DNA is pelleted by centrifugation, dried and dissolved in a large volume (0.1 ng/μl) of TE. After ligation the mixtures were transformed to *E. coli*. Plasmid DNA isolated from these transformants was analyzed by restriction analysis to reveal the right constructs. In this way the deletion constructs pPRGDH3ΔHIII, pPRGDH6ΔBamHI, pPRGDH6ΔSstI and pPRGDH6ΔSalI (Fig. 1).

In addition to this, the 0.6-kb and 0.8-kb *Sst*I fragments derived from pPRGDH6 were subcloned in the corresponding site of pUC19.

Sequence analysis was carried out using pUC/M13 forward and reverse primers (Promega). The sequencing strategy is shown in fig. 2 (see arrows).

On the basis of homology with the GAPDH-gene sequence of *S. cerevisiae* (Holland and Holland, 1979. J. of Biol. Chem. 254: 9839-9845) and *K. lactis* (Shuster, 1990. Nucl. Acids Res. 18, 4271) and the known splice site consensus J.L. Woolford. 1989. Yeast 5: 439-457), the introns and the possible ATG start were postulated.

The GAPDH gene has 6 introns (Fig. 1) and encodes a polypeptide of 339 amino acids. This was completely unexpected considering the genomic organisation of the GAPDH genes of *K. lactis* and *S. cerevisiae* which have no introns and both consist of 332 amino acids. The homology on the amino acid level between the GAPDH gene of *Phaffia* and *K. lactis* and *S. cerevisiae* is 63% and 61%, respectively.

Most of the introns in the GAPDH gene are situated at the 5' part of the gene. Except intron III all introns contain a conserved branch-site sequence 5'-CTPuAPy-3' found for *S. cerevisiae* and *S. pombe*.

By computer analysis of the upstream sequence using PC-gene 2 putative eukaryotic promoter elements, TATA-box (position 249-263 in SEQIDNO: 11) and a number of putative Cap signal (between 10 position 287 and 302 in SEQIDNO: 11) were identified.

Example 5

Cloning of the GAPDH promoter fused to G418 in pUCG418.

In order to construct a transcription fusion between the GAPDH promoter and the gene encoding G418 resistance the fusion PCR technique was used.

Using plasmid pPRGDH6 the GAPDH promoter could be amplified by standard PCR protocols ("Experimental").

In the PCR mix pPRGDH6 and oligo's No. 5177 and 5126 (Sequences in "Experimental") were used. A 416 bp DNA fragment was generated containing the entire GAPDH promoter sequence. In addition this fragment also contains a *Hind*III, *Xba*I and a *Kpn*I restriction site at it's 5'end and 12 nt overlap with the 5' end of the gene encoding G418 resistance.

The 217 bp portion of the 5'end of the G418 coding sequence was also amplified by PCR using pUC-G418 and oligo's 4206 and 5127. A 226 bp DNA fragment was obtained containing the 217 bp 5'end of G418 and having a 9 nucleotides overlap with the 3'end of the earlier generated GAPDH promoter fragment. It also contained a *Msc*I site at it's 3end.

The PCR fragments were purified from the PCR mixture using the WIZARD Kit.

Approximately 1 μ g of the GAPDH promoter fragment and 1 μ g of the G418 PCR fragment were used together with oligo's 5177 and 4206 in a fusion PCR experiment (Experimental). A 621 bp DNA fragment was generated, containing the GAPDH promoter directly fused to the 5' portion of G418. After purification the DNA fragment was digested with *Msc*I and *Kpn*I. The 3.4 Kb *Msc*I-*Kpn*I fragment of pUC-G418, containing pUC sequences and the 3' portion of G418, was used as a vector.

The ligation mixture was transformed to competent *E. coli* DH5 α cells. Transformant colonies containing the fusion PCR DNA inserted were identified by digestion with different restriction enzymes.

Thus, plasmid pPR1 was obtained, containing the GAPDH promoter directly fused to the G418 marker gene. Three pPR1 vectors isolated from independent transformants were used in further cloning experiments.

To target the plasmid, after transformation, to a specific integration site a 3.0-kb *Sst*I fragment containing a part of the ribosomal DNA of *Phaffia* was cloned in pPR1. The ribosomal DNA fragment was isolated from an agarose gel after digestion with *Sst*I of plasmid pGB-Ph11 (EP 590 707 A1). This

fragment was ligated in the dephosphorylated SstI site of pPR1. The ligation mixture was transformed to competent *E. coli* cells. Plasmid DNA was isolated and using restriction analysis it was shown that several colonies contain the expected plasmid pPR2. The complete cloning strategy is shown in Fig. 3.

5 Example 6

Transformation of *Phaffia* with pPR2.

Transformation of *Phaffia* strain 6938 was performed using an electroporation procedure as previously described by Faber et al. (1994, Curr. Genet. 1994: 25,305-310) with the following modifications:

- 10 - Electropulsing was performed using the Bio-rad Gene Pulser with Pulse Controller and with Bio-rad 2mm cuvettes.
- *Phaffia* was cultivated for 16 h at 21 °C.
- Per transformation 2×10^8 cells were used together with 5 µg of linearized vector. Linearization was done in the rDNA sequence using *Cla*I to enable integration at the rDNA locus in the *Phaffia* genome.
- 15 Following the electric pulse (7.5kV/cm, 400 Ω and 25 µF) 0.5 ml YePD medium was added to the cell/DNA mixture. The mixture was incubated for 2.5 h at 21 °C and subsequently spread on 5 selective YEDP agar plates containing 40 µg/ml G418.

As shown in Table 2 we were able to generate transformants with 115 transformants per µg DNA; the average transformation frequency was 50 transformants/µg pPR2 as judged over a number of 20 experiments. Transformation of the closed circular form of pPR2 did not result in transformation suggesting that there is no autonomously replicating sequence present within the vector sequences. Using pPR2 a 10 to 50-fold increase in transformation frequency was found compared to a previous constructed transformation vector for *Phaffia*, called pGB-Ph9. In this latter vector a translation fusion was made between the 5' part of the actin gene of *Phaffia* and G418.

25 In order to analyze the level of resistance of transformants the mixture of DNA/cells was plated onto selective plates containing different amounts of G418. Although the total number of transformants decreases with the increasing amounts of G418, we were still able to obtain a considerable number of transformants (table 3).

In another experiment 30 transformants obtained under standard selection conditions (40 µg/ml) 30 were transferred to plates containing 50, 200 or 1000 µg/ml. After incubation of the plates at 21 °C for 4-5 days, 23 transformants out of 30 tested were able to grow on plates containing 200 µg/ml G418. One transformant was able to grow on plates containing up to and above 1000 µg/ml G418.

35 Table 2.

Transformation frequency of pGB-Ph9 and pPR2.

	Exp. 1	Exp. 2
--	69	8
pGB-Ph9xBg/II	46	7
40 pPR2 ccc	n.d	n.d
pPR2(A)x <i>Cla</i> I	714	56
(B)	639	124

(C)

443

153

5 Total number of transformants (> 1 mm) in different transformation experiments after 4-5 days incubation.

Table 3. Comparison of G418 sensitivity as a result of two different G418-resistance genes in pGB-Ph9 and pPR2

10	concentration G418 (µg/ml)	Number of transformants	
		pPR2xClaI	pGB-Ph9xBglII (=pYac4)
15	40	480	2
	50	346	-
	60	155	-
	70	61	-
	80	141	-
20	90	72	-
	100	64	-

Analysis of pPR2 transformants.

25 To analyse the integration event and the number of integrated vector copies total genomic DNA from six independent transformants was isolated. Therefore these transformants were cultivated under selective conditions, *i.e.* YePD + 50 µg/ml G418. Chromosomal DNA was digested with *Cla*I. The DNA fragments were separated by gel electrophoresis and transferred to nitrocellulose and the Southern blot was probed with *Phaffia* DNA.

30 Besides the rDNA band of 9.1 kb an additional band of 7.1 kb of similar fluorescing intensity was observed in the transformants. This band corresponds to the linearised form of pPR2. From the intensity of these bands it was concluded that the copy number was about 100 - 140 copies of pPR2. These results are similar to those observed for pGB-Ph9, ruling out that the improved G418-resistance is due to differences in copy number of integrated vectors alone. It is not known whether the multiple copy event is caused by multiple copy integration of pPR2 or by the amplification of a single copy in the 35 rDNA or a combination of both events.

Example 7

Construction of pPR2T by cloning the GAPDH-terminator into pPR2

35 Eukaryotic mRNAs contain modified terminal sequences, specifically the 3' terminal poly(A). As the prokaryotic gene encoding G418 resistance lacks eukaryotic termination signals, which might effect proper transcription termination and mRNA stability (1994, Raue, H.A., TIBTECH 12: 444-449), a part of the 3' non-coding sequence of GAPDH was introduced.

To that end, a 307 bp fragment, consisting of 281 bp of the 3' non-coding region of GAPDH and other additional cloning sequences, was amplified by PCR using the oligo's 5137 and 5138 ("Experimental"). 45 The upstream oligo 5137 consists of the last 14 nucleotides of the coding and 17 nucleotides of the 3' non-coding region of GAPDH. By base substitutions of the 5th (T --> A) and 8th (T --> C) nucleotide

of the non-coding sequence a *Bam*HI restriction site was introduced. In addition this fragment contains a *Xba*I and a *Hind*III restriction site at its 3' end.

The PCR fragment was purified from the PCR mixture using the WIZARD Purification Kit and digested with *Bam*HI and *Hind*III. A 288 bp fragment was isolated and cloned into the corresponding sites of the previously constructed *Phaffia* transformation vector pPR2, yielding pPR2T.

Upon transformation of *Phaffia*, using G418 as selective agent, the transformation frequencies (number of transformants per μ g of DNA) obtained with the improved construct pPR2T was approximately 5 to 10 times higher than the transformation frequency of pPR2 (i.e. without a *Phaffia* homologous transcription termination signal). The results of a typical experiment are given in Table 4.

10

Table 4 Transformation frequency at 50 μ g/ml G418 for pGB-Ph9, pPR2 and pPR2T

Vector	transformants	transformants/ μ g DNA
pGB-Ph9 (ccc)	-	-
pGB-Ph9 (x <i>Bgl</i> II)	60	1
15 pPR2 (ccc)	1	-
pPR2 (x <i>Cla</i> I)	3000 - 9600	50 - 160
pPR2T (ccc)	-	-
pPR2T (x <i>Cla</i> I)	45600	760
pPR2T (x <i>Sfi</i> I)	1080	18

20

Phaffia cells transformed with pPR2T were tested for their ability to grow on high levels of G418. The level of G418 on which growth is still possible was taken as a measure of the expression level of the G418 resistance gene in transformants, as a result of the presence of the *Phaffia* promoter, and/or terminator. Preliminary results indicate that the number of transformants able to grow on high levels of G418 are significantly higher than without terminator.

In summary

From the above results, it was concluded, that the presence of the GAPDH-promoter (pPR2) resulted in a considerable increase of the transformation frequency (from 1 to at least 50 per μ g of DNA) when compared to the vector containing the actin-promoter (pGB-Ph9). These results are in line with the results obtained with the G418 sensitivity test (Table 3 and 4) which indicate superior expression levels under the control of the GAPDH promoter. The possibility that the difference in transformation frequency could be due solely to the difference in linearising the vectors, (*Bgl*II, *Cla*I and *Sfi*I all cut inside the ribosomal DNA locus, but at different positions), was ruled out by comparison of pPR2(x*Sfi*I) with pGB-Ph9(x*Sfi*I). The difference in transformation frequency between the two pPR2 and pGB-Ph9, linearised with *Sfi*I is still considerable. However, it is concluded that the choice of the linearisation site does have effect on the transformation frequency; linearisation with *Cla*I is preferred.

The improvements obtained by using a high-level promoter, such as GAPDH, are irrespective of whether a homologous terminator is used (pPR2 (without homologous terminator) performs far better than pGB-Ph9, both in G418 sensitivity tests, as well as in terms of transformation frequency).

The presence of a homologous terminator results in both higher transformation frequencies and higher expression levels; this result is concluded to be independent of the promoter used. Preliminary results indicate that considerable improvements are obtained when the pGB-Ph9 construct is completed with a transcription terminator, such as the GAPDH-terminator used in pPR2T.

The following Examples illustrate the isolation of DNA encoding enzymes involved in the carotenoid biosynthesis pathway of *Phaffia rhodozyma*. These DNA sequences can suitably be used for a variety of purposes; for example to detect and isolate DNA sequences encoding similar enzymes in other organisms, such as yeast by routine hybridisation procedures, to isolate the transcription promoters and/or terminators, which can be used to construct expression vectors for both heterologous as well as homologous downstream sequences to be expressed. The DNA sequences encoding carotenoid biosynthesis genes can suitably be used to study the over-expression, either under the control of their own promoters or heterologous promoters, such as the glycolytic pathway promoters illustrated above. For example, transformation of *Phaffia rhodozyma* with carotenoid encoding DNA sequences according to the invention effectively results in amplification of the gene with respect to the wild-type situation, and as a consequence thereof to overexpression of the encoded enzyme.

Hence, the effect of over-expression of one or more genes encoding carotenoid biosynthesis genes can thus be studied. It is envisaged that mutant *Phaffia* strains can be obtained producing higher amounts of valuable carotenoids, such as β -carotene, canthaxanthin, zeaxanthin and/or astaxanthin. Similarly, the DNA sequences encoding enzymes involved in the carotenoid biosynthesis pathway can be introduced into other hosts, such as bacteria, for example *E. coli*, yeasts, for example species of *Saccharomyces*, *Kluyveromyces*, *Rhodosporidium*, *Candida*, *Yarrowia*, *Phycomyces*, *Hansenula*, *Pichia*, fungi, such as *Aspergillus*, *Fusarium*, and plants such as carrot, tomato, and the like. The procedures of transformation and expression requirements are well known to persons skilled in these arts.

Strains: *E. coli* XL-Blue-MRF' $\Delta(mcrA)183\Delta(mcrCB-hsdSMR-mrr)$ 173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F' proAB laq^qZ Δ M15 Tn10 (Tet^r)]*
ExAssistTM interference-resistant helper phage (Stratagene^R)
P. rhodozyma CBS6938 or
P. rhodozyma asta 1043-3

Plasmids used for cloning:

pUC19 Ap^r (Gibco BRL)
Uni-ZAPTM XR vector (lambda ZAP^R II vector digested with *Eco*RI-*Xba*I, CIAP treated; Stratagene^R)

Media: LB: 10 g/l bacto tryptone, 5 g/l yeast extract, 10 g/l NaCl. Plates: +20 g/l bacto agar.

When appropriate 50-100 µg/ml ampicillin (Ap), 30 µg/ml chloramphenicol (Cm) and 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added.

YPD: 10 g/l yeast extract, 20 g/l bacto peptone, 20 g/l glucose. Plates: +20 g/l bacto agar.

5 All molecular cloning techniques were essentially carried out as described by Sambrook et al. in Molecular Cloning: a Laboratory Manual, 2nd Edition (1989; Cold Spring Harbor Laboratory Press). Transformation of *E. coli* was performed according to the CaCl₂ method described by Sambrook et al.

Enzyme incubations were performed following instructions described by the manufacturer. These incubations include restriction enzyme digestion, dephosphorylation and ligation (Gibco BRL).

10 Isolation of plasmid DNA from *E. coli* was performed using the QIAGEN (Westburg B.V. NL).

For sequence analysis deletions constructs and oligonucleotides were made to sequence the complete sequence using a *Taq* DYE Primer Cycle Sequencing kit (Applied Biosystems).

Example 8

15 Description of plasmids

Plasmids (pACCAR25ΔcrtE, pACCAR25ΔcrtB, pACCRT-EIB, pACCAR16ΔcrtX and pACCAR25ΔcrtX), which contain different combinations of genes involved in the biosynthesis of carotenoid in *Erwinia uredovora* were gifts from Prof. Misawa; Kirin Brewery co.,LTD.; Japan). The biosynthetic route of carotenoid synthesis in *Erwinia uredovora* is shown in fig 8.

20 In addition a derivative of pACCAR25ΔcrtX, designated pACCAR25ΔcrtXΔcrtI, was made in our laboratory. By the introduction of a frameshift in the *Bam*H1 restriction site the crtI gene was inactivated. *E. coli* strains harboring this plasmid accumulate phytoene which can be monitored by the red phenotype of the colony.

All plasmids are derivatives of plasmid pACYC184 (Rose RE; Nucl. Acids Res. 16 (1988) 355), which 25 contains a marker conferring chloramphenicol-resistance. Furthermore these plasmids and derivatives thereof contain a replication origin that is compatible to vectors such as pUC and pBluescript. Each plasmid contains a set of carotenoid biosynthetic genes of *Erwinia uredovora* mediating the formation of different carotenoid in *E. coli*. The complete list of plasmid used in this study is shown in Table 5.

30 Table 5: Summary of carotenoid producing *E. coli* strains used in this study.

PLASMID:	GENOTYPE:	CAROTENOID ACCUMULATED:	COLOR PHENOTYPE:
pACCAR25ΔcrtE	<i>crtB</i> ; <i>crtI</i> ; <i>crtY</i> ; <i>crtX</i> ; <i>crtZ</i>	farnesyl pyrophosphate/iso-pentenyl pyrophosphate	white
pACCAR25ΔcrtB	<i>crtE</i> ; <i>crtI</i> ; <i>crtY</i> ; <i>crtX</i> ; <i>crtZ</i>	geranylgeranyl pyrophosphate	white
35 pACCAR25ΔcrtX ΔcrtI	<i>crtE</i> ; <i>crtB</i> ; <i>crtY</i> ; <i>crtZ</i>	phytoene	white

pACCRT-E1B	<i>crtE; crtB; crtI</i>	lycopene	red
pACCAR16Δ <i>crtX</i>	<i>crtE; crtB; crtI; crtY</i>	β-carotene	yellow
pACCAR25Δ <i>crtX</i>	<i>crtE; crtB; crtI; crtY; crtZ</i>	zeaxanthin	yellow/orange

10 Genes encoding: *crtE*, geranylgeranyl pyrophosphate synthase; *crtB*, Phytoene synthase; *crtI*, phytoene desaturase; *crtY*, lycopene cyclase; *crtX*, β-carotene hydroxylase; *crtZ*, zeaxanthin glycosylase

Example 9

Construction of cDNA library of *Phaffia rhodozyma*

15 a) Isolation of total RNA from *Phaffia rhodozyma*

All solutions were made in DEPC-treated distilled water and all equipments were soaked overnight in 0.1% DEPC and then autoclaved.

20 A 300 ml Erlenmeyer containing 60 ml YePD culture medium was inoculated with *Phaffia rhodozyma* strain CBS6938/1043-3 from a preculture to a final OD₆₀₀ of 0.1. This culture was incubated at 21 °C (300 rpm) until the OD₆₀₀ had reached 3-4.

25 The cells were harvested by centrifugation (4 °C, 8000 rpm, 5 min) and were resuspended in 12 ml of ice-cold extraction-buffer (0.1 M Tris-HCl, pH 7.5; 0.1 M LiCl; 0.1 mM EDTA). After centrifugation cells were resuspended in 2 ml of ice-cold extraction-buffer, 4 g of glassbeads (0.25 mm) and 2 ml phenol were added.

The mixture was vortexed 5 times at maximum speed for 30 s with 30 s cooling incubation intervals on ice.

30 The cell/glassbeads/phenol mixture was centrifuged (5 min, 15.300 rpm, 4 °C) and the aqueous phase (sup 1) was transferred to a fresh tube and was kept on ice.

35 The phenolic phase was retracted by adding an additional volume of 1 ml extraction buffer and 2 ml phenol.

After centrifugation (5 min, 15.300 rpm, 4 °C), the aqueous phase was transferred to sup 1 and extracted with an equal volume phenol:chloroform.

40 After centrifugation (5 min, 15.300 rpm, 4 °C), the aqueous phase was transferred to a fresh tube and 0.1 volume of 3 M NaAc; pH 5.5 and 2.5 volumes of EtOH was added to precipitate RNA (incubation overnight -20 °C).

45 The precipitate was collected by centrifugation (10 min, 15.300 rpm, 4 °C) and drained off excess liquid and the RNA pellet was washed with 70 % icecold EtOH.

After removing excess liquid the RNA was resuspended in 200 - 800 μ l DEPC-treated water. RNA was stored at -70 °C. A 60 ml culture yielded 400 - 1500 μ g total RNA. The integrity of total RNA was checked by formaldehyde RNA gel electrophoresis.

5 b) Selection of poly(A)* RNA

Isolation of poly(A)* from total RNA was carried out essential as described by Sambrook et al., 1989 (Molecular cloning, a laboratory manual, second edition) using the following solutions.

All solutions were prepared in DEPC-treated water and autoclaved.

10 RNA denaturation buffer: 1 M NaCl; 18% (v/v) DMSO.

Column-loading buffer (HEND): 10 mM Hepes, pH 7.6; 1 mM EDTA; 0.5 M Na Cl; 9% (v/v) DMSO.

Elution buffer (HE): 10 mM Hepes, pH 7.6; 1 mM EDTA.

Oligo(dT)-cellulose Type 7 was supplied by Pharmacia Biotech. 0.1 g (dry weight) of oligo(dT)-cellulose was add to 1 ml HEND and the suspension was gently shaked for 1 h at 4 °C. Total RNA (1.5 mg dissolved in 500 μ l) and 1 ml 1 M NaCl; 18% (v/v) DMSO was heated to 65 °C for 5 min. Then 600 μ l NaCl/DMSO was added to the RNA, mixed and placed on ice for 5 min. The poly(A)* isolation was carried out be two cycles of purification. The final yield was about 45 μ g poly(A)* RNA.

20 c) cDNA synthesis

cDNAs were synthesized from 7.5 μ g poly(A)*-RNAs using the cDNA Synthesis Kit (#200401; Strategene[®]). Synthesis was carried out according to the instruction manual with some minor modification.

25 SuperScriptTM II RNase H⁻ Reverse Transcriptase (Gibco BRL) was used in the first strand reaction instead of MMLV-RT.

The following reagents were add in a microcentrifuge:

3 μ l of poly(A)* RNAs

2 μ l of linker-primer

23.5 μ l DMQ

30 Incubate 10 min 70 °C, spin quickly in microcentrifuge and add,

10 μ l of 5 x First Strand Buffer (provided by Gibco BRL)

5 μ l of 0.1 M DTT (provided by Gibco BRL)

3 μ l of first strand methyl nucleotide mixture

1 μ l of RNase Block Ribonuclease Inhibitor (40 U/ μ l)

35 Annealling of template and primers by incubation the mixture at 25 °C for 10 min followed by 2 min at 42 °C and finally add:

2.5 μ l SuperScriptTM II RNase H⁻ Reverse Transcriptase

First-strand reaction was carried out at 42 °C for 1 h.

Size fractionation was carried out using Geneclean® II kit (supplied BIO 101, Inc.). The volume of the cDNA mixture obtained after *Xba*I digestion was brought up by adding DMQ to a final volume of 200 µl. Three volumes of NaI was added and the microcentrifuge tube was placed on ice for 5 min. The pellet of glassmilk was washed three times using 500 µl New Wash. Finally the cDNA was eluted in 20 µl DMQ.

The yield of cDNA was about 1 µg using these conditions.

d) cDNA cloning

10 cDNA library was constructed in the Uni-ZAP™ XR vector using 100 ng cDNAs. Ligation was performed two times overnight incubation at 12 °C. The cDNA library was packaged using the Packagene® lambda DNA packaging system (Promega) according to the instruction manual. The calculated titer of the cDNA library was 3.5 10⁶ pfu.

15 **e) Mass excision**

Mass excision was carried out described in the protocol using derivatives of *E. coli* XL-Blue-MRF' as acceptor strain (see Table 5). Dilution of cell mixtures were plated onto 145 mm LB agar plates containing ampicillin, chloramphenicol and IPTG, yielding 250 - 7000 colonies on each plate. The plates 20 were incubated overnight at 37 °C and further incubated one or two more days at room temperature.

Example 10

Cloning of the geranylgeranyl pyrophosphate synthase gene (*crtE*) of *Phaffia rhodozyma*

25 **a) Isolation of cDNA clone**

The entire library was excised into a farnesylpyrophosphate/ isopentenyl pyrophosphate accumulating cells of *E. coli* XL-Blue-MRF, which carries the plasmid pACCAR25ΔcrtE (further indicated as XL-Blue-MRF'[pACCAR25ΔcrtE]). The screening for the *crtE* gene was based on the color of the 30 transformants. Introduction of the *crtB* gene in a genetic background of XL-Blue-MRF'[pACCAR25ΔcrtE] would result in a restoration of the complete route for the biosynthesis of zeaxanthin-diglucoside, which could be monitored by the presence of a yellow/orange pigmented colony. About 8.000 colonies were spread on LB agar plates containing appropriate antibiotics and IPTG. One colony was found to have changed to a yellow/orange color.

35 **b) Characterization of complementing cDNA clone**

These colonies were streaked on LB-ampicillin agar plates. Plasmid DNA was isolated from this yellow colonies and found to include a 1.85 kb fragment (Fig 2A). The resulting plasmid, designated pPRcrtE,

was used for retransformation experiments (Table 6). Only the transformation of XL-Blue-MRF'[pACCAR25ΔcrtE] with pPRcrtE resulted in a white to yellow color change in phenotype. To test whether the color change was due to complementation and not caused by cDNA alone pPRcrtE was transformed into XL-Blue-MRF'. Selection of transformants on LB-ampicillin agar plate containing IPTG did not result in color changes of the colonies (Table 6). Therefore we tentatively concluded, that we have cloned a cDNA of *P. rhodozyma* encoding GPPP synthase which is involved in the conversion of IPP and FPP to GGPP.

Table 6: Color phenotype of carotenoid producing *E. coli* strains transformed with pPRcrtE.

	pUC19 (control)	pPRcrtE
XL-Blue-MRF' (Ap, IPTG)	white	white
XL-Blue-MRF' [pACCAR25ΔcrtE] (Ap, Cm, IPTG)	white	yellow/orange
XL-Blue-MRF' [pACCAR25ΔcrtB] (Ap, Cm, IPTG)	white	white

Transformation: 10 ng of each plasmid was mixed to CaCl_2 competent *E. coli* cells. Transformant cells were selected by plating 1/10 and 1/100 volume of the DNA/cell mixture on LB agar-medium containing the appropriate antibiotics (in brackets).

c) Sequence analysis of cDNA fragment

Plasmid pPRcrtE was used to determine the nucleotide sequence of the 1.85 kb cDNA. The sequence comprised 1830 nucleotides and a 31 bp poly(A) tail. An open reading frame (ORF) of 375 amino acids was predicted. The nucleotide sequence and deduced amino acid sequence are shown as SEQIDNO: NO 14 and 15, respectively. A search in SWISS-PROT protein sequence data bases using the Blitz amino acid sequence alignment program indicated amino acid homology (52 % in 132 aa overlap; *Neurospora crassa*) especially to the conserved domain I in geranylgeranyl-PPi synthase enzymes of different organisms (Botella et al., Eur. J. Biochem. (1995) 233; 238-248).

Example 11

Cloning of the phytoene synthase gene (crtB) of *Phaffia rhodozyma*

a) Isolation of cDNA clone

The entire library was excised into a geranylgeranylpyrophosphate accumulating cells of *E. coli* XL-Blue-MRF', which carries the plasmid pACCAR25ΔcrtB (further indicated as XL-Blue-MRF'[pACCAR25ΔcrtB]). The screening for the crtB gene was based on the color of the transformants.

Introduction of the *crtB* gene in a genetic background of XL-Blue-MRF'[pACCAR25Δ*crtB*] would result in a restoration of the complete route for the biosynthesis of zeaxanthin-diglucoside, which could be monitored by the presence of a yellow/orange pigmented colony.

About 25.000 colonies were incubated on LB agar plates containing appropriate antibiotics and IPTG.

5 Three colonies were found to have changed to a yellow/orange color.

b) Characterization of complementing cDNA clone

These colonies were streaked on LB-ampicillin agar plates. Plasmid DNA, designated pPR*crtB*1 to 3, 10 was isolated from these yellow colonies and found to include a 2.5 kb fragment (Fig 2B). One of the resulting plasmids, pPR*crtB*1 was used for retransformation experiments (Table 7). Only the transformation of XL-Blue-MRF'[pACCAR25Δ*crtB*] with pPR*crtB* resulted in a white to yellow color 15 change in phenotype. Therefore we tentatively conclude that we have cloned a cDNA of *P. rhodozyma* encoding phytoene synthase which is involved in the conversion of 2 GGPP molecules via prephytoene pyrophosphate into phytoene.

Table 7: Color phenotype of carotenoid producing *E. coli* strains transformed with pPR*crtB*.

	pUC19 (control)	pPR <i>crtB</i>
20	XL-Blue-MRF' (Ap, IPTG)	white
25	XL-Blue-MRF' [pACCAR25Δ <i>crtB</i> (Ap, Cm, IPTG)]	yellow/orange
	XL-Blue-MRF' [pACCAR25Δ <i>crtE</i> (Ap, Cm, IPTG)]	white

Legend: see Table 6.

30 c) Sequence analysis of cDNA fragment.

Plasmid pPR*crtB*2, which contains the longest cDNA insert, was used to determine the nucleotide sequence of the 2.5 kb cDNA. The sequence comprised 2483 nucleotides and a 20 bp poly(A) tail. An 35 open reading frame (ORF) of 684 amino acids was predicted. The nucleotide sequence and deduced amino acid sequence are shown in SEQIDNOs: 12 and 13, respectively. A search in SWISS-PROT protein sequence data bases using the Blitz amino acid sequence alignment program Data indicated some amino acid homology (26 % identity in 441 aa overlap of *crtB* gene of *Neurospora crassa*) with *crtB* genes of other organisms.

40

Example 12

Cloning of the phytoene desaturase gene (*crtI*) of *Phaffia rhodozyma*

a) Isolation of cDNA clone

The entire library was excised into a phytoene accumulating cells of *E.coli* XL-Blue-MRF', which carries the plasmid pACCAR25ΔcrtXΔcrtI (further indicated as XL-Blue-MRF'[pACCAR25ΔcrtXΔcrtI]). The screening for the *crtI* gene was based on the color of the transformants. Introduction of the *crtI* gene in a genetic background of XL-Blue-MRF'[pACCAR25ΔcrtXΔcrtI] would result in a restoration of the complete route for the biosynthesis of zeaxanthin, which could be monitored by the presence of a yellow/orange pigmented colony.

About 14.000 colonies were incubated on LB agar plates containing appropriate antibiotics and IPTG. Two colonies were found to have changed to a yellow/orange color.

10

b) Characterization of complementing cDNA clones

These colonies were streaked on LB-ampicillin agar plates. Plasmid DNA, designated pPRcrtI.1 and pPRcrtI.2, was isolated from these yellow colonies and found to include a 2.0 kb fragment (Fig 2C). 15 One of the resulting plasmids, pPRcrtI.1 was used for retransformation experiments (Table 8). Only the transformation of XL-Blue-MRF'[pACCAR25ΔcrtXΔcrtI] with pPRcrtI resulted in a white to yellow color change in phenotype. Therefore we tentatively conclude that we have cloned a cDNA of *P. rhodozyma* encoding phytoene desaturase which is involved in the conversion of phytoene to lycopene.

20

Table 8: Color phenotype of carotenoid producing *E. coli* strains transformed with pPRcrtI.

	pUC19	pPRcrtI
XL-Blue-MRF' (Ap, IPTG)	white	white
XL-Blue-MRF' [pACCAR25ΔcrtX ΔcrtI (Ap, Cm, IPTG)]	white	yellow/orange
XL-Blue-MRF' [pACCAR25ΔcrtB (Ap, Cm, IPTG)]	white	white

Legend: see Table 6.

35 c) Sequence analysis of cDNA fragment

One of the plasmid pPRcrtI was used to determine the nucleotide sequence of the 2.0 kb cDNA. The sequence comprised 2038 nucleotides and a 20 bp poly(A) tail. An open reading frame (ORF) of 582 amino acids was predicted. The nucleotide sequence and deduced amino acid sequence are shown in 40 SEQIDNOs: 16 and 17, respectively. A search in SWISS-PROT protein sequence data bases using the Blitz amino acid sequence alignment program Data indicated amino acid homology to phytoene desaturase gene of *N. crassa* (53% identity in 529 aa overlap).

Example 13Cloning of the lycopene cyclase gene (*crtY*) of *Phaffia rhodozyma*a) Isolation of cDNA clone

5 The entire library was excised into a lycopene accumulating cells of *E.coli* XL-Blue-MRF', which carries the plasmid pACCRT-EIB (further indicated as XL-Blue-MRF'[pACCRT-EIB]). The screening for the *crtY* gene was based on the color of the transformants. Introduction of the *crtY* gene in a genetic background of XL-Blue-MRF'[pACCRT-EIB] would result in a restoration of the complete route for the biosynthesis of β -carotene, which could be monitored by the presence of a yellow pigmented colony.

10 About 8.000 colonies were incubated on LB agar plates containing appropriate antibiotics and IPTG. One colony was found to have changed to a yellow color.

b) Characterization of complementing cDNA clone

15 This colony was streaked on LB-ampicillin agar plates. Plasmid DNA was isolated from this yellow colony and found to include a 2.5 kb fragment (Fig 2B). The resulting plasmid, designated pPRcrtY, was used for retransformation experiments (Table 9. Surprisingly, not only transformation of XL-Blue-MRF'[pACCRT-EIB] but also transformation of XL-Blue-MRF'[pACCAR25 Δ crtB] with pPRcrtY resulted in a red to yellow color change in phenotype.

20

Table 9: Color phenotype of carotenoid producing *E. coli* strains transformed with pPRcrtY.

	pUC19	pPRcrtY
XL-Blue-MRF' (Ap, IPTG)	white	white
XL-Blue-MRF' [pACCRT-EIB (Ap, Cm, IPTG)	red	yellow
XL-Blue-MRF' [pACCAR25 Δ crtB (Ap, Cm, IPTG)	red	yellow

Legend: see Table 6.

A second transformation experiment was carried out including the previously cloned cDNA of pPRcrtB.

35 As shown in table 6 the cDNA previously (example 3) isolated as encoding phytoene synthase was able to complement the *crtY* deletion resulting in the biosynthesis of β -carotene in XL-Blue-MRF'[pACCRT-EIB].

Sequence analysis of the cDNA insert of pPRcrtY (SEQIDNOs: 18 and 19) showed that it was similar to the sequence of cDNA fragment of pPRcrtB.

From these data we tentatively conclude that we have cloned a cDNA of *P. rhodozyma* encoding phytoene synthase and lycopene cyclase which is involved in the conversion of 2 GGPP molecules via 5 prephytoene pyrophosphate into phytoene and lycopene to β -carotene, respectively. This is the first gene in a biosynthetic pathway of carotenoids synthesis that encodes two enzymatic activities.

Table 10: Color phenotype of carotenoid producing *E. coli* strains transformed with different cDNAs of *Phaffia rhodozyma* (Ap, Cm, IPTG).

	pUC19	pPRcrtE	pPRcrtB	pPRcrtY
XL-Blue-MRF' [pACCAR25ΔcrtE]	white	yellow/orange	white	white
XL-Blue-MRF' [pACCAR25ΔcrtB]	white	white	yellow/orange	yellow/orange
XL-Blue-MRF' [pACCRT-EIB]	red	red	yellow	yellow

Legend: see Table 6

Example 14

Cloning of the isopentenyl diphosphate (IPP) isomerase gene (idi) of *Phaffia rhodozyma*

a) Isolation of cDNA clone

The entire *Phaffia* cDNA library was excised into lycopene accumulating cells of *E. coli* XL-Blue-MRF', each carrying the plasmid pACCRT-EIB (further indicated as XL-Blue-MRF'[pACCRT-EIB]).

About 15.000 colonies were incubated on LB agar plates containing appropriate antibiotics and IPTG. One colony was found to have a dark red colour phenotype.

b) Characterization of complementing cDNA clone

This colony was streaked on LB-ampicillin agar plates. Plasmid DNA was isolated from this yellow colony and found to include a 1.1 kb fragment. The resulting plasmid, designated pPRcrtX, was used for 35 retransformation experiments (Table 11).

All colonies of XL-Blue-MRF'[pACCAR-EIB] transformed with pPRcrtX had a dark red phenotype. From these data we tentatively concluded, that we have cloned a cDNA of *P. rhodozyma* expression of which results in an increased lycopene production in a genetically engineered *E. coli* strain.

Table 11: Color phenotype of carotenoid producing *E. coli* strains transformed with pPRcrtX.

	pUC19	pPRcrtX
XL-Blue-MRF' (Ap, IPTG)	white	white
XL-Blue-MRF' [pACCRT-EIB (Ap, Cm, IPTG)]	red	dark red

Legend: see Table 6.

10 c) Sequence analysis of cDNA fragment

In order to resolve the nature of this gene the complete nucleotide sequence of the cDNA insert in pPRcrtX was determined. The nucleotide sequence consist of the 1144 bp. The sequence comprised 1126 nucleotides and a poly(A) tail of 18 nucleotides. An open reading frame (ORF) of 251 aminoacids with 15 a molecular mass of 28.7 kDa was predicted. The nucleotide sequence and deduced amino acid sequence are shown in SEQIDNOs: 20 and 21, respectively.

15 A search in SWISS-PROT protein sequence data bases using the Blitz amino acid sequence alignment program Data indicated aminoacid homology to isopentenyldiphosphate (IPP) isomerase (*idi*) of *S. cerevisiae* (42.2 % identity in 200 aminoacid overlap). IPP isomerase catalyzes an essential activation 20 step in the isoprene biosynthetic pathway which synthesis the 5-carbon building block of carotenoids. In analogy to yeast the gene of *Phaffia* was called *idi1*. The cDNA clone carrying the genes was then called pPR*idi*.

Example 15

25 Overexpression of the *idi* gene of *P. rhodozyma* in a carotenogenic *E. coli*

Lycopene accumulating cells of *E.coli* XL-Blue-MRF', which carry the plasmid pACCRT-EIB (further indicated as XL-Blue-MRF'[pACCRT-EIB]) were transformed with pUC19 and pPR*idi* and 30 transformants were selected on solidified LB-medium containing Amp and Cm. The transformants, called XL-Blue-MRF'[pACCRT-EIB/pUC19 and [pACCRT-EIB/pPR*idi*], were cultivated in 30 ml LB-medium containing Amp, Cm and IPTG at 37 °C at 250 rpm for 16 h. From these cultures 1 ml was used for carotenoid extraction and analysis. After centrifugation the cell pellet was dissolved in 200 µl aceton and incubated at 65 °C for 30 minutes. Fifty µl of the cell-free aceton fraction was then used for high-performance liquid chromatography (HPLC) analysis. The column (chrompack cat. 28265; packing 35 nucleosil 100C18) was developed with water-acetonitrile-2-propanol (from 0 to 45 minutes 9:10:81 and after 45 minutes 2:18:80) at a flow rate of 0.4 ml per minute and recorded with a photodiode array detector at 470 +/- 20 nm. Lycopene was shown to have a retention time of about 23 minutes under these conditions. The peak area was used as the relative lycopene production (mAu*s). The relative

lycopene production was 395 and 1165 for XL-Blue-MRF'[pACCRT-EIB/pUC19] and [pACCRT-EIB/pPR*idi*], respectively.

These data show the potentials of metabolic pathway engineering in *Phaffia*, as increased expression of the *idi* of *Phaffia rhodozyma* causes a 3-fold increase in carotenoid biosynthesis in *E. coli*.

5 This cDNA may be over-expressed in a transformed *Phaffia* cell with a view to enhance carotenoid and/or xanthophyll levels. The cDNA is suitably cloned under the control of a promoter active in *Phaffia*, such as a strong promoter according to his invention, for example a *Phaffia* glykolytic pathway promoter, such as the GAPDH-gene promoter disclosed herein, or a *Phaffia* ribosomal protein gene promoter according to the invention (*vide sub*). Optionally, the cDNA is cloned in front of a
10 transcriptional terminator and/or polyadenylation site according to the invention, such as the GAPDH-gene terminator/polyadenylation site. The feasibility of this approach is illustrated in the next example, where the *crtB* gene from *Erwinia uredovora* is over-expressed in *Phaffia rhodozyma* by way of
15 illustration.

15

Example 16

Heterologous expression of carotenogenic gene from *Erwinia uredovora* in *Phaffia rhodozyma*.

The coding sequence encoding phytoene synthase (*crtB*) of *Erwinia uredovora* (Misawa et al., 1990) was cloned between the promoter and terminator sequences of the *gpd* (GAPDH-gene) of *Phaffia* by fusion PCR. In two separate PCR reactions the promoter sequence of *gpd* and the coding sequence of *crtB* were amplified. The former sequence was amplified using the primers 5177 and 5128 and pPR8 as template. This latter vector is a derivative of the *Phaffia* transformation vector pPR2 in which the promoter sequence of *gpd* was amplified by PCR using the primers 5226 and 5307 and plasmid pPRgpd6 as template. The amplified promoter fragment was isolated, digested with *Kpn*I and *Bam*HI and cloned in 20 the *Kpn*I-*Bg*II fragment of vector pPR2, yielding pPR8. The coding sequence of *crtB* was amplified using the primers 5131 and 5134 and pACCRT-EIB as template. In a second fusion PCR reaction, using the primers 5177 and 5134, 1 µg of the amplified promoter and *crtB* coding region fragment used as template yielding the fusion product Pgpd-*crtB*. The terminator sequence was amplified under standard PCR conditions using the primers 5137 and 5138 and the plasmid pPRgpd6 as template. Primer 5137 25 contains at the 5' end the last 11 nucleotides of the coding region of the *crtB* gene of *E. uredovora* and the first 16 nucleotides of the terminator sequence of *gpd* gene of *P. rhodozyma*. By a two basepair substitution a *Bam*HI restriction site was introduced. The amplified fusion product (Pgpd-*crtB*) and the amplified terminator fragments were purified and digested with *Hind*III and *Bam*HI and cloned in the dephosphorylated *Hind*III site of the cloning vector pMTL25. The vector with the construct Pgpd-*crtB*-
30 *Tgpd* was named pPREX1.1.
35

The *Hind*III fragment containing the expression cassette Pgpd-*crtB*-*Tgpd* was isolated from pPREX1.1 and ligated in the dephosphorylated *Hind*III site of the *Phaffia* transformation vector pPR8. After transformation of the ligation mixture into *E. coli* a vector (pPR8-*crtB*6.1) with the correct insert was chosen for *Phaffia* transformation experiments.

Phaffia strain CBS6938 was transformed with pPR8crtB6.1, carrying the expression cassette Pgpd-crtB-Tgpd, and transformants were selected on plates containing G418. The relative amount of astaxanthin per OD₆₆₀ in three G418-resistant transformants and the wild-type *Phaffia* strains was determined by HPLC analysis (Table 12). For carotenoid isolation from *Phaffia* the method of DMSO/hexane extraction described by Sedmak *et al.*, (1990; *Biotechn. Techniq.* 4, 107-112) was used.

Table 12. The relative astaxanthin production in a *Phaffia* transformant carrying the *crtB* gene of *E. uredovora*.

10	Relative amount	of astaxanthin
	Strain:	(mAU*s/OD ₆₆₀)
15	<i>P. rhodozyma</i> CBS6938	448
	<i>P. rhodozyma</i> CBS6938	
	[pPR8crtB6.1]#1	626
	[pPR8crtB6.1]#2	716
	[pPR8crtB6.1]#4	726

20 Primers used:

5128: 5' *caactgc*ca*gtatggtaagaggttttagag* 3'
 5177: 5' cccaagctttccgaggtacctggtgggtcatgtatgtac 3'
 25 5131: 5' taccateatggcagttggctgaaaag 3'
 5134: 5' cccaagctttgcatcgttgtctagagcggggcctgcc 3'
 5137: 5' ccaaggcctaaaacggatccctccaaacc 3'
 5138: 5' gccaagcctccgagttgtgatcagataaagatagat 3'
 5307: 5' gttgaagaaggggatcttgtggatga 3'

30

The *gpd* sequences are indicated in bold, the *crtB* sequences in italic, additional restriction sites for cloning are underlined and base substitution are indicated by double underlining.

Example 17

35 Isolation and characterization of the *crtB* gene of *Phaffia*

It will also be possible to express the *Phaffia rhodozyma* gene corresponding to *crtB* and express it under the control of its own regulatory regions, or under the control of a promoter of a highly expressed gene according to the invention. The *Phaffia* transformation procedure disclosed herein, invariably leads to stably integrated high copy numbers of the introduced DNA, and it is expected, that 40 expression of the gene under the control of its own promoter will also lead to enhanced production of

carotenoids, including astaxanthin. To illustrate the principle, a prot col is given for the cloning of the *crtB* genomic sequence, below.

To obtain the genomic *crtB*-gene including expression signals the 2.5 kb *Bam*HI-*Xba*I fragment was isolated from the vector pPRcrtB and used as probe to screen a cosmid library of *Phaffia*.

5 The construction and screening of the library was carried out as described in Example 3 using the *crtB* gene as probe instead of the *gapdh*-gene.

After the rounds of hybridization, 2 colonies were identified giving a strong hybridization signal on the autoradiogram after exposure. Cosmid DNA isolated from these colonies was called pPRgcrtB#1.1 and pPRgcrtB#7, respectively.

10 Chromosomal DNA isolated from *Phaffia rhodozyma* strain CBS 6938 and cosmid pPRgcrtB#7 was digested with several restriction enzymes. The DNA fragments were separated, blotted and hybridized with a amino-terminal specific probe (0.45 kb *Xba*I fragment) of *crtB* under conditions as described before. After exposure, the autoradiogram showed DNA fragments of different length digested by different restriction enzymes which hybridized with the *crtB* probe. On the basis that no *Eco*RI site is 15 present in the cDNA clone a *Eco*RI fragment of about 4.5 kb was chosen for subcloning experiments in order to determine the sequence in the promoter region and to establish the presence of intron sequences in the *crtB* gene. A similar sized hybridizing fragment was also found in the chromosomal DNA digested with *Eco*RI. The fragment was isolated from an agarose gel and ligated into the corresponding site of pUC19. The ligation mixture was transformed to competent *E. coli* cells. Plasmids with the correct insert 20 in both orientations, named pPR10.1 and pPR10.2, were isolated from the transformants. Comparison of the restriction patterns of pPR10.1/pPR10.2 and pPRcrtB digested with *Xba*I gave an indication for the presence of one or more introns as the internal 2.0 kb *Xba*I fragment in the cDNA clone was found to be larger in the former vectors. The subclone pPR10.1 was used for sequence analysis of the promoter 25 region and the structural gene by the so-called primer walking approach. The partial sequence of the insert is shown in SEQIDNO: 22. Comparison of the cDNA and the genomic sequence revealed the presence of 4 introns.

Example 18

Isolation of promoter sequences with high expression levels

30 This example illustrates the the feasibility of the "cDNA sequencing method" referred to in the detailed description, in order to obtain transcription promoters from highly expressed genes.

For the isolation and identification of transcription promoter sequences from *Phaffia rhodozyma* genes exhibiting high expression levels, the cDNA library of *Phaffia rhodozyma* was analyzed by the following procedure.

35 The cDNA library was plated on solidified LB-medium containing Amp and 96 colonies were randomly picked for plasmid isolation. The purified plasmid was digested with *Xba*I and *Xba*I and loaded on a agarose gel. The size of the cDNA inserts varied from 0.5 to 3.0 kb. Subsequently, these plasmids were used as template for a single sequence reaction using the T3 primer. For 17 cDNA clones no sequence data were obtained. The sequences obtained were translated in all three reading frames. For

each cDNA sequence the longest deduced amino acid sequences were compared with the SwissProt protein database at EBI using the Blitz program. For 18 deduced amino acid sequences no homology to known proteins was found whereas six amino acid sequences showed significant homology to hypothetical proteins. Fifty-five amino acid sequences were found to have significant homology to proteins for which the function is known. About 50 % (38/79) were found to encode ribosomal proteins of which 12 full-length sequences were obtained.

Table 13. Overview of expressed cDNAs, encoded proteins and reference to the Sequence Listing

10	cDNA	coding for	SEQIDNO:
10	10	ubiquitin-40S	24
11	11	Glu-repr.gene	26
15	18	40S rib.prot S27	28
35	35	60S rib.prot P1 α	30
38	38	60S rib.prot L37e	32
46	46	60S rib.prot L27a	34
64	64	60S rib.prot L25	36
20	68	60S rib.prot P2	38
73	73	40S rib.prot S17A/B	40
76	76	40S rib.prot S31	42
78	78	40S rib.prot S10	44
85	85	60S rib.prot L37A	46
25	87	60S rib.prot L34	48
95	95	60S rib.prot S16	50

By sequence homology it was concluded that in *Phaffia* the 40S ribosomal protein S37 is fused to ubiquitin as is found in other organisms as well. The nucleotide sequences and deduced amino acid sequences of the full length cDNA clones are listed in the sequence listing. Six ribosomal proteins were represented in the random pool by more than one individual cDNA clone. The 40S ribosomal proteins S10 (SEQIDNO:44), S37 (+ ubiquitin) (SEQIDNO:24) and S27 (SEQIDNO:28) were represented twice and 60S (acidic) ribosomal proteins P2 (SEQIDNO:38), L37 (SEQIDNO:46) and L25 (SEQIDNO:36) found three times. From these results we conclude, that these proteins are encoded by multiple genes or that these genes are highly expressed. Therefore isolation of these promoter sequences are new and promising target sequences to isolate high level expression signals from *Phaffia rhodozyma*. Furthermore, a cDNA clone was isolated which showed 50 % homology to an abundant glucose-repressible gene from *Neurospora crassa* (Curr. genet. 14: 545-551 (1988)). The nucleotide sequence and the deduced amino acid sequence is shown in SEQIDNO:26. One of the advantages of such a promoter sequence is that it can be used to separate growth (biomass accumulation) and gene expression (product accumulation) in large scale *Phaffia* fermentation.

For the isolation of the promoter sequences of interest (as outlined above) a fragment from the corresponding cDNA clone can be used as probe to screen the genomic library of *Phaffia rhodozyma* following the approach as described for the GAPDH-gene promoter (Example 3, *supra*). Based on the determined nucleotide sequence of the promoter, specific oligonucleotides can be designed to construct a transcription fusion between the promoter and any gene of interest by the fusion PCR technique, following the procedure as outlined in Example 5 (*supra*).

Example 19

Isolation of carotenogenic genes by heterologous hybridization

For the identification and isolation of corresponding carotenoid biosynthetic pathway genes from organisms related to *Phaffia rhodozyma* heterologous hybridization experiments were carried out under conditions of moderate stringency. In these experiments chromosomal DNA from two carotenogenic fungi (*Neurospora crassa* and *Blakeslea trispora*) and the yeasts *S. cerevisiae* and three yeast and fungal species from the genus *Cystofylobasidium* was used. These three carotenogenic yeasts are, based on phylogenetic studies, the ones most related to *P. rhodozyma*.

Chromosomal DNA from the yeast species *Cystofylobasidium infirmo-miniatum* (CBS 323), *C. bisporidii* (CBS 6346) and *C. capitatum* (CBS 6358) was isolated according the method as developed for *Phaffia rhodozyma*, described in example 3 of European patent application 0 590 707 A1; the relevant portions of which herein incorporated by reference. Isolation of chromosomal DNA from the fungi *Neurospora crassa* and *Blakeslea trispora* was essentially carried as described by Kolar et al. (Gene, 62: 127-134), the relevant parts of which are herein incorporated by reference.

Chromosomal DNA (5 µg) of *C. infirmo-miniatum*, *C. bisporidii*, *C. capitatum*, *S. cerevisiae*, *P. rhodozyma*, *N. crassa* and *B. trispora* was digested using *EcoRI*. The DNA fragments were separated on a 0.8% agarose gel, blotted and hybridized using the following conditions.

Hybridization was carried out at two temperatures (50 °C and 55 °C) using four different ³²P labelled *Phaffia* probes. The probes were made using random primed hexanucleotide labellings reactions using the *XbaI-XbaI* fragment(s) from the cDNA clones pPRcrtE, pPRcrtB, pPRcrtI and pPRidi as template. Hybridization was carried out o/n (16 h) at the indicated temperatures. After hybridization the filters were washed 2 times for 30 min. at the hybridization temperatures using a solution of 3*SSC; 0.1 % SDS; 0.05% sodiumpyrophosphate. Films were developed after exposure of the filters to X-ray films in a cassette at -80 °C for 20 h.

Using the cDNA clone of *crtE* of *P. rhodozyma* faint signals were obtained for *C. infirmo-miniatum*, *C. capitatum*. Using the cDNA clone of *crtB* of *P. rhodozyma* strong signals were obtained to the high molecular weight portion of DNA from *C. infirmo-miniatum* and *C. capitatum*. Furthermore a strong signal was obtained in the lane loaded with digested chromosomal DNA from *B. trispora*. Only a faint signal was obtained for *C. capitatum* at 50 °C using the cDNA clone of *crtI* of *P. rhodozyma*. Using the cDNA clone of *idi* of *P. rhodozyma* faint signals were obtained with chromosomal DNA from *C. infirmo-miniatum*, *C. bisporidii* and *C. capitatum* at both temperatures. A strong signal was obtained in the lane loaded with digested chromosomal DNA from *B. trispora*.

We conclude, that carotenoid biosynthesis cDNAs or genes, or *idi* cDNAs or genes, can be isolated from other organisms, in particular from other yeast species by cross-hybridisation with the cDNA fragments coding for *P. rhodozyma* carotenoid biosynthesis enzymes, or isopentenyl pyrophosphate isomerase coding sequences respectively, using moderately stringent hybridisation and washing conditions (50 °C to 55 °C, 3xSSC).

Deposited microorganisms

E. coli containing pGB-Ph9 has been deposited at the Centraal Bureau voor Schimmelcultures, Oosterstraat 1, Baarn, The Netherlands, on June 23, 1993, under accession number CBS 359.3.

10 The following strains have been deposited under the Budapest Treaty at the Centraal Bureau voor Schimmelcultures, Oosterstraat 1, Baarn, The Netherlands, on February 26, 1996:

ID nr.	Organism	relevant feature	Deposit number
DS31855	<i>E. coli</i>	<i>criY</i> of <i>P. rhodozyma</i>	CBS 232.96
DS31856	<i>E. coli</i>	<i>criI</i> of <i>P. rhodozyma</i>	CBS 233.96
15 DS31857	<i>E. coli</i>	<i>criE</i> of <i>P. rhodozyma</i>	CBS 234.96
DS31858	<i>E. coli</i>	<i>criB</i> of <i>P. rhodozyma</i>	CBS 235.96

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT:

10

- (A) NAME: Gist-brocades B.V.
- (B) STREET: Wateringseweg 1
- (C) CITY: Delft
- (E) COUNTRY: The Netherlands
- (F) POSTAL CODE (ZIP): 2611 XT

15

(ii) TITLE OF INVENTION: Improved methods for transforming *Phaffia* and recombinant DNA for use therein

20

(iii) NUMBER OF SEQUENCES: 51

25

(iv) COMPUTER READABLE FORM:

30

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

35

(v) CURRENT APPLICATION DATA:

40

APPLICATION NUMBER:

(2) INFORMATION FOR SEQ ID NO:1:

45

(i) SEQUENCE CHARACTERISTICS:

50

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

55

(ii) MOLECULE TYPE: DNA (genomic)

60

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

65

- (C) INDIVIDUAL ISOLATE: AB3005

70

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

75

CGGGATCCAA RCTNACNGGN ATGGC

25

80

(2) INFORMATION FOR SEQ ID NO:2:

85

(i) SEQUENCE CHARACTERISTICS:

90

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

95

(ii) MOLECULE TYPE: DNA (genomic)

100

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

105

- (C) INDIVIDUAL ISOLATE: AB3006

110

(ix) FEATURE:

115

- (A) NAME/KEY: misc_feature
- (B) LOCATION: one-of(12)
- (D) OTHER INFORMATION: /note= "N at position 12 is inosine"

120

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGGGATCCRT ANCCVYAYTC RITRTRCTAC CA

32

(2) INFORMATION FOR SEQ ID NO:3:

5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

15 (vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: AB4206

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

20 GCGTGACTTC TGGCCAGCCA CGATAGC

27

(2) INFORMATION FOR SEQ ID NO:4:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

35 (vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: AB5126

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

40 TTCAAATCCAC ATGATGGTAA GAGTGTAGA GA

32

(2) INFORMATION FOR SEQ ID NO:5:

45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

55 (vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: AB5127

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

60 CTTACCATCA TGTGGATTGA ACAAGATGGA T

31

(2) INFORMATION FOR SEQ ID NO:6:

65 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

70

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: AB5177

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

10 CCCAAGCTTC TCGAGGTACC TGGTGGGTGC ATGTATGTAC

40

(2) INFORMATION FOR SEQ ID NO:7:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: AB5137

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

30 CCAAGGCCAA AAAOGGATCC CTCCAAACCC

30

(2) INFORMATION FOR SEQ ID NO:8:

35 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: AB5138

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

50 GCGAAGCTTC TCGAGCTTGA TCAAGATAAAG ATAGAGAT

38

(2) INFORMATION FOR SEQ ID NO:9:

55 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2309 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

60 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Phaffia rhodozyma*
- (B) STRAIN: CBS 6938

5 (ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 300..330

10 5 (ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 331..530

15 10 (ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 531..578

20 15 (ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 579..668

25 20 (ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 669..690

30 25 (ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 691..767

35 30 (ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 768..805

40 35 (ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 806..905

45 40 (ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 906..923

50 50 (ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 924..1030

55 45 (ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1031..1378

60 55 (ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1379..1508

65 60 (ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1509..2020

70 65 (ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: join(300..330, 531..578, 669..690, 768..805, 906..923, 1031..1378, 1509..2020)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

60	GCTATGAGCA AGCACAACTG GGCACCGAAC GAGAACAGTA ACTGTGGGTA TCTTCCACC	60
	GACACGAGGC GTCTCCCGGC GGCAACCGCC GGTGGCCCGCC TCGCTTAAAG TCAGCCACCC	120
65	AGTTTTCTTC CATCTCTTTC TCTCTCTTTC CAAAGTCCTT TCAGTTTAA AGGGCCCCCA	180
	AAAAAAAGAAG AGGGGACTTT TCTTTCCCTT CTCCCCATCA TCCACAAAGA TCCTCTCTCT	240
70	TCAACAACTAA CTACTACTAC TACCACTACC ACCACTACTT CTCTAACACT CTTACCATC	299

ATG GCT GTC AAG GTT GGA ATC AAC GGT TTC G	GTATGIGTTT GTTTTCTCT	350
Met Ala Val Lys Val Gly Ile Asn Gly Phe		
1 5 10		
5 TGAGCTCCCC CATCGTTCTT TTOGCTTGTG CAGTTTCIT TTTCCCTTCC TTTCTTTTC		410
TTTTTCTCC CCACTGCCIT TTTTTTTCTT ATTCCTTTTT TTTTCCCTTC CTCCTGCTT		470
10 CATGCAATGC ACTAACACCA TCTCATCTCA TCTCACTCTG CCTCGCTTA CCTCTACAG		530
GA CGA ATC GGA CGA ATC GTC CTT CGA AAC GCT ATC ATC CAC GGT GAT A		578
Gly Arg Ile Gly Arg Ile Val Leu Arg Asn Ala Ile Ile His Gly Asp		
15 20 25		
15 GTCAGTATTT TTTTAATTTC TTTTTTCC CATCAATTTC CCTCTGCTCC TTTACTCATC		638
TCTTTCCATC TCTCTCCAC TCTCCCTACAG TC GAT GTC GTC GCC ATC AAC GA		690
Ile Asp Val Val Ala Ile Asn Asp		
30		
20 GIGCGTCTAG ATCGACCATC TCGTGTGCG CCCAACACC GTCTGACACC ATCCTGTTAA		750
CTTTCTCTC CTCCAAG C CCT TTC ATC GAT CTT GAG TAC ATG GTC TAC ATG		801
Pro Phe Ile Asp Leu Glu Tyr Met Val Tyr Met		
35 40 45		
25 TTC A GTCAGTCTCC CTCCCCCTCA AAAAGCCGAA ACAAAAGCCGA ACAGAACCG		855
Phe		
10 ATCTAACCAT TCGTTCTCTC TCCCTTCCCT CTTCCGTCCTC TCCCTCACAG AG TAC		910
Lys Tyr		
35 GAC TCC ACC CAC G GTTCGTCAT CCTCTCTCTC GTCCCCAACCA TCTCCGACCG		963
Asp Ser Thr His		
50		
40 GGCCTTTCC ATCTCCCTGAT CGGTTGGGT ACTAACCCAT ACGTACCCCT TGGTCCATC		1023
CCTTCAG GT GTC TTC AAG GGA TCC GTC GAG ATC AAG GAC GGC AAG CTC		1071
Gly Val Phe Lys Gly Ser Val Glu Ile Lys Asp Gly Lys Leu		
55 60 65		
45 GTG ATC GAG GGC AAG CCC ATC GTC GTC TAC GGT GAG CGA GAC CCC GGC		1119
Val Ile Glu Gly Lys Pro Ile Val Val Tyr Gly Glu Arg Asp Pro Ala		
70 75 80		
50 AAC ATC CAG TGG GGA GCT GCC GGT GCC GAC TAC GTC GTC GAG TCC ACC		1167
Asn Ile Gln Trp Gly Ala Ala Gly Ala Asp Tyr Val Val Glu Ser Thr		
85 90 95		
55 GGT GTC TTC ACC ACC CAG GAG AAG GCC GAG CTC CAC CTC AAG GGA GGA		1215
Gly Val Phe Thr Thr Gln Glu Lys Ala Glu Leu His Leu Lys Gly Gly		
100 105 110		
60 GCC AAG AAG GTC ATC TCT GCC CCT TCG GCC GAT GCC CCC ATG TTC		1263
Ala Lys Lys Val Val Ile Ser Ala Pro Ser Ala Asp Ala Pro Met Phe		
115 120 125 130		
65 GTC TGC GGT GTT AAC CTC GAC AAG TAC GAC CCC AAG TAC ACC GTC GTC		1311
Val Cys Gly Val Asn Leu Asp Lys Tyr Asp Pro Lys Tyr Thr Val Val		
135 140 145		
70 TCC AAC GCT TCG TGC ACC ACC AAC TGC TTG GCT CCC CTC GGC AAG GTC		1359
Ser Asn Ala Ser Cys Thr Thr Asn Cys Leu Ala Pro Leu Gly Lys Val		
150 155 160		
75 ATC CAC GAC AAC TAC ACC A GTCAAGTCCTT TNCCTTGGAC TTGTCCTGGCC		1408

Ile His Asp Asn Tyr Thr
165

TTTTCTTTGT TGGTCTTTT CCTTTTGCA ACCATCCAT ACTCACCCCTG TTTTCACCT 1468
5 TCCTTTCTCT CATTCAGTA TCCCCCTCC CGTCCACCAAG TT GTC GAG GGT CTC Ile Val Glu Gly Leu 1522
170

ATG ACC ACC GTC CAC GCC ACC ACC GCC ACC CAG AAG ACC GTC GAC GGT 1570
10 Met Thr Thr Val His Ala Thr Ala Thr Gln Lys Thr Val Asp Gly 175 180 185

CCT TCC AAC AAG GAC TGG CGA GGA GGT CGA GGA GCT GGT GCC AAC ATC 1618
15 Pro Ser Asn Lys Asp Trp Arg Gly Gly Arg Gly Ala Gly Ala Asn Ile 190 195 200 205

ATT CCC TCC ACC GGA GCC GCC AAG GCC GTC GGT AAG GTT ATC CCC 1666
20 Ile Pro Ser Ser Thr Gly Ala Ala Lys Ala Val Gly Lys Val Ile Pro 210 215 220

TCC CTC AAC GGA AAG CTC ACC GGA ATG GCC TTC CGA GTG CCC ACC CCC 1714
25 Ser Leu Asn Gly Leu Thr Gly Met Ala Phe Arg Val Pro Thr Pro 225 230 235

GAT GTC TCC GTC GTC GAT CTT GTC GTC CGA ATC GAG AAG GGC GCC TCT 1762
30 Asp Val Ser Val Val Asp Leu Val Val Arg Ile Glu Lys Gly Ala Ser 240 245 250

TAC GAG GAG ATC AAG GAG ACC ATC AAG AAG GCC TCC CAG ACC CCT GAG 1810
35 Tyr Glu Glu Ile Lys Glu Thr Ile Lys Lys Ala Ser Gln Thr Pro Glu 255 260 265

CTC AAG GGT ATC CTG AAC TAC ACC GAC GAC CAG GTC GTC TCC ACC GAT 1858
40 Leu Lys Gly Ile Leu Asn Tyr Thr Asp Asp Gln Val Val Ser Thr Asp 270 275 280 285

TTC ACC GGT GAC TCT GCC TCC ACC TTC GAC GCC CAG GGC GGT ATC 1906
45 Phe Thr Gly Asp Ser Ala Ser Ser Thr Phe Asp Ala Gln Gly Ile 290 295 300

TCC CTT AAC GGA AAC TTC GTC AAG CTT GTC TCC TGG TAC GAC AAC GAG 1954
50 Ser Leu Asn Gly Asn Phe Val Lys Leu Val Ser Trp Tyr Asp Asn Glu 305 310 315

TGG GGA TAC TCT GCC CGA GTC TGC GAC CTT GTT TCT TAC ATC GCC GCC 2002
55 Trp Gly Tyr Ser Ala Arg Val Cys Asp Leu Val Ser Tyr Ile Ala Ala 320 325 330

CAG GAC GCC AAG GCC TAAACGGGTC TCTCCAAACC CTCTCCCTT TTGCCCTGCC 2057
60 Gln Asp Ala Lys Ala 335

CATTGAATTG ATTCCCTAAA TAGAATATCC CACTTCTTT TATGCTCTAC CTATGATCAG 2117
65 TTATCTGTC TTTTCTTGTG TGGGTTGCGG TTGTCGACT GTACCCACCT CTTGAGGGAC 2177

AAGGCAAGAA GTGAGCAAGA TATGAACAAG AACAAACAAAG AAAAGAGAC AAAGAAAAAA 2237
70 AAAAGGAAAG AGAAAACAAT CCCCOCCCCC CCCCAAAAAA AAATCTCTAT CTTTATCTGA 2297
TCAAGAGATT AT 2309

65 (2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 338 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

1 Met Ala Val Lys Val Gly Ile Asn Gly Phe Gly Arg Ile Gly Arg Ile
 5 10 15
 Val Leu Arg Asn Ala Ile Ile His Gly Asp Ile Asp Val Val Ala Ile
 20 25 30
 10 Asn Asp Pro Phe Ile Asp Leu Glu Tyr Met Val Tyr Met Phe Lys Tyr
 35 40 45
 15 Asp Ser Thr His Gly Val Phe Lys Gly Ser Val Glu Ile Lys Asp Gly
 50 55 60
 Lys Leu Val Ile Glu Gly Lys Pro Ile Val Val Tyr Gly Glu Arg Asp
 65 70 75 80
 20 Pro Ala Asn Ile Gln Trp Gly Ala Ala Gly Ala Asp Tyr Val Val Glu
 85 90 95
 Ser Thr Gly Val Phe Thr Thr Gln Glu Lys Ala Glu Leu His Leu Lys
 25 100 105 110
 Gly Gly Ala Lys Lys Val Val Ile Ser Ala Pro Ser Ala Asp Ala Pro
 115 120 125
 30 Met Phe Val Cys Gly Val Asn Leu Asp Lys Tyr Asp Pro Lys Tyr Thr
 130 135 140
 Val Val Ser Asn Ala Ser Cys Thr Thr Asn Cys Leu Ala Pro Leu Gly
 145 150 155 160
 35 Lys Val Ile His Asp Asn Tyr Thr Ile Val Glu Gly Leu Met Thr Thr
 165 170 175
 Val His Ala Thr Thr Ala Thr Gln Lys Thr Val Asp Gly Pro Ser Asn
 40 180 185 190
 Lys Asp Trp Arg Gly Arg Gly Ala Gly Ala Asn Ile Ile Pro Ser
 195 200 205
 45 Ser Thr Gly Ala Ala Lys Ala Val Gly Lys Val Ile Pro Ser Leu Asn
 210 215 220
 Gly Lys Leu Thr Gly Met Ala Phe Arg Val Pro Thr Pro Asp Val Ser
 225 230 235 240
 50 Val Val Asp Leu Val Val Arg Ile Glu Lys Gly Ala Ser Tyr Glu Glu
 245 250 255
 Ile Lys Glu Thr Ile Lys Lys Ala Ser Gln Thr Pro Glu Leu Lys Gly
 55 260 265 270
 Ile Leu Asn Tyr Thr Asp Asp Gln Val Val Ser Thr Asp Phe Thr Gly
 275 280 285
 60 Asp Ser Ala Ser Ser Thr Phe Asp Ala Gln Gly Gly Ile Ser Leu Asn
 290 295 300
 Gly Asn Phe Val Lys Leu Val Ser Trp Tyr Asp Asn Glu Trp Gly Tyr
 65 305 310 315 320
 Ser Ala Arg Val Cys Asp Leu Val Ser Tyr Ile Ala Ala Gln Asp Ala
 325 330 335
 70 Lys Ala

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 388 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

15 (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Phaffia rhodozyma*

(ix) FEATURE:

- (A) NAME/KEY: promoter
- (B) LOCATION:1..385

20 (ix) FEATURE:

- (A) NAME/KEY: TATA signal
- (B) LOCATION:249..263

25 (D) OTHER INFORMATION:/label= putative

(ix) FEATURE:

- (A) NAME/KEY: misc_signal
- (B) LOCATION:287..302

30 (D) OTHER INFORMATION:/function= "cap-signal"
/label= putative

(ix) FEATURE:

- (A) NAME/KEY: misc_RNA
- (B) LOCATION:386..388

35 (D) OTHER INFORMATION:/function= "start of CDS"

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION:85

40 (D) OTHER INFORMATION:/note= "uncertain"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

45	TCGTGGGTGC ATGTAATGTC GIGAGTGAGT GGGGGGAAA GGGGAGTACG TGTGTTGTCAGC	60
	CGCAAGGAAG AACTAACGAG CGCAGCTAT GAGCAAGCAC AACTGGGCAC CGAACGAGAA	120
50	CAGTAACTGT CGGTATCTTC CCACCGACAC GAGGCGTCAC CGGGGGCAA CGGGGGTGC	180
	CCCCCTCGC TTACGTCAGC CACCCAGTTT CTTCACATCT CTTCCTCTCT CCTTCACAAA	240
	GTCTTTCAGT TTTAAACGGC CCCCAAAAAA AGAAGAGGGG ACTTTTCTT TCCTTCCTTC	300
55	CCATCATCCA CAAAGATCTC TCTTCCTCAA CAACAACTAC TACTACTACC ACTACCACCA	360
	CTACTTCCTCT AACACTCTTA CCATCATG	388

60 (2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2546 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

70 (ii) MOLECULE TYPE: cDNA

	(iii) HYPOTHETICAL: NO	
	(iv) ANTI-SENSE: NO	
5	(vi) ORIGINAL SOURCE:	
	(A) ORGANISM: <i>Phaffia rhodozyma</i>	
10	(ix) FEATURE:	
	(A) NAME/KEY: CDS	
	(B) LOCATION: 225..2246	
	(D) OTHER INFORMATION: /product= "PRcrtB"	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:	
15	TCTAGAACTA GTGGATCCCC CGGGCTGCAG GAATTCGGCA CGAGCGGAAA CAAGAAGTCG	60
	ACACAGAGAG ATCTTTGCAG AAGAGTTGTA TTCCAGAAAG GGAAACAAA GGAAGAAGC	120
	GGCGAAGCAC ATCACCAACT TCAGCAGGCC GGTCCAGGCC GATCTGGAT AGCATCATC	180
20	TTACCCAACT CGTATCATCC CCAACAGATA GAGTTTTGT CGCA ATG ACC GCT CTC	236
	Met Thr Ala Leu	
	1	
25	GCA TAT TAC CAG ATC CAT CTG ATC TAT ACT CTC CCA ATT CTT GGT CTT	284
	Ala Tyr Tyr Gln Ile His Leu Ile Tyr Thr Leu Pro Ile Leu Gly Leu	
	5 10 15 20	
30	CTC GGC CTG CTC ACT TCC CGG ATT TTG ACA AAA TTT GAC ATC TAC AAA	332
	Leu Gly Leu Leu Thr Ser Pro Ile Leu Thr Lys Phe Asp Ile Tyr Lys	
	25 30 35	
35	ATA TCG ATC CTC GTC ATT GCG TTT AGT GCA ACC ACA CCA TGG GAC	380
	Ile Ser Ile Leu Val Phe Ile Ala Phe Ser Ala Thr Thr Pro Trp Asp	
	40 45 50	
40	TCA TGG ATC ATC AGA AAT GGC GCA TGG ACA TAT CCA TCA GCG GAG AGT	428
	Ser Trp Ile Ile Arg Asn Gly Ala Trp Thr Tyr Pro Ser Ala Glu Ser	
	55 60 65	
45	GGC CAA GGC GTG TTT GGA ACG TTT CTA GAT GTT CCA TAT GAA GAG TAC	476
	Gly Gln Gly Val Phe Gly Thr Phe Leu Asp Val Pro Tyr Glu Glu Tyr	
	70 75 80	
50	GCT TTC TTT GTC ATT CAA ACC GTC ATC ACC GGC TTG GTC TAC GTC TTG	524
	Ala Phe Phe Val Ile Gln Thr Val Ile Thr Gly Leu Val Tyr Val Leu	
	85 90 95 100	
55	GCA ACT AGG CAC CTT CTC CCA TCT CTC GCG CTT CCC AAG ACT AGA TCG	572
	Ala Thr Arg His Leu Leu Pro Ser Leu Ala Leu Pro Lys Thr Arg Ser	
	105 110 115	
60	TCC GCC CTT TCT CTC GCG CTC AAG GCG CTC ATC CCT CTG CCC ATT ATC	620
	Ser Ala Leu Ser Leu Ala Leu Lys Ala Leu Ile Pro Leu Pro Ile Ile	
	120 125 130	
65	TAC CTA TTT ACC GCT CAC CCC AGC CCA TCG CCC GAC CGG CTC GTG ACA	668
	Tyr Leu Phe Thr Ala His Pro Ser Pro Ser Pro Asp Pro Leu Val Thr	
	135 140 145	
70	GAT CAC TAC TTC TAC ATG CGG GCA CTC TCC TTA CTC ATC ATC ACC CCA CCT	716
	Asp His Tyr Phe Tyr Met Arg Ala Leu Ser Leu Leu Ile Thr Pro Pro	
	150 155 160	
75	ACC ATG CTC TTG GCA GCA TTA TCA GGC GAA TAT GCT TTC GAT TGG AAA	764
	Thr Met Leu Leu Ala Ala Leu Ser Gly Glu Tyr Ala Phe Asp Trp Lys	
	165 170 175 180	
80	AGT GGC CGA GCA AAG TCA ACT ATT GCA GCA ATC ATG ATC CGG ACG GTG	812
	Ser Gly Arg Ala Lys Ser Thr Ile Ala Ala Ile Met Ile Pro Thr Val	

	185	190	195	
5	TAT CTG ATT TGG GTA GAT TAT GTT GCT GTC GGT CAA GAC TCT TGG TCG Tyr Leu Ile Trp Val Asp Tyr Val Ala Val Gly Gln Asp Ser Trp Ser 200 205 210			860
10	ATC AAC GAT GAG AAG ATT GTA GGG TGG AGG CTT GGA GGT GTA CTA CCC Ile Asn Asp Glu Lys Ile Val Gly Trp Arg Leu Gly Gly Val Leu Pro 215 220 225			908
15	ATT GAG GAA GCT ATG TTC TTC TTA CTG ACG AAT CTA ATG ATT GTT CTG Ile Glu Glu Ala Met Phe Phe Leu Leu Thr Asn Leu Met Ile Val Leu 230 235 240			956
20	GGT CTG TCT GCC TGC GAT CAT ACT CAG GCC CTA TAC CTG CTA CAC GGT Gly Leu Ser Ala Cys Asp His Thr Gln Ala Leu Tyr Leu Leu His Gly 245 250 255 260			1004
25	CGA ACT ATT TAT GGC AAC AAA AAG ATG CCA TCT TCA TTT CCC CTC ATT Arg Thr Ile Tyr Gly Asn Lys Lys Met Pro Ser Ser Phe Pro Leu Ile 265 270 275			1052
30	ACA CGG CCT GTG CTC TCC CTG TTT TTT AGC AGC CGA CCA TAC TCT TCT Thr Pro Pro Val Leu Ser Leu Phe Ser Ser Arg Pro Tyr Ser Ser 280 285 290			1100
35	CAG CCA AAA CGT GAC TTG GAA CTG GCA GTC AAG TTG TTG GAG AAA AAG Gln Pro Lys Arg Asp Leu Glu Leu Ala Val Lys Leu Leu Glu Lys Lys 295 300 305			1148
40	AGC CGG AGC TTT TTT GTT GCC TCG GCT GGA TTT CCT AGC GAA GTT AGG Ser Arg Ser Phe Phe Val Ala Ser Ala Gly Phe Pro Ser Glu Val Arg 310 315 320			1196
45	GAG AGG CTG GTT GGA CTA TAC GCA TTC TGC CGG GTG ACT GAT GAT CTT Glu Arg Leu Val Gly Leu Tyr Ala Phe Cys Arg Val Thr Asp Asp Leu 325 330 335 340			1244
50	ATC GAC TCT CCT GAA GTA TCT TCC AAC CGG CAT GCC ACA ATT GAC ATG Ile Asp Ser Pro Glu Val Ser Ser Asn Pro His Ala Thr Ile Asp Met 345 350 355			1292
55	GTC TCC GAT TTT CTT ACC CTA CTA TTT GGG CCC CGG CTA CAC CCT TCG Val Ser Asp Phe Leu Thr Leu Leu Phe Gly Pro Pro Leu His Pro Ser 360 365 370			1340
60	CAA CCT GAC AAG ATC CTT TCT TCG CCT TTA CTT CCT OCT TCG CAC CCT Gln Pro Asp Lys Ile Leu Ser Ser Pro Leu Leu Pro Pro Ser His Pro 375 380 385			1388
65	TCC CGA CCC ACG GGA ATG TAT CCC CTC CGG CCT CCT CCT TCG CTC TCG Ser Arg Pro Thr Gly Met Tyr Pro Leu Pro Pro Pro Ser Leu Ser 390 395 400			1436
70	CCT GCC GAG CTC GTT CAA TTC CTT ACC GAA AGG GTT CCC GTT CAA TAC Pro Ala Glu Leu Val Gln Phe Leu Thr Glu Arg Val Pro Val Gln Tyr 405 410 415 420			1484
75	CAT TTC GCC TTC AGG TTG CTC GCT AAG TTG CAA GGG CTG ATC CCT CGA His Phe Ala Phe Arg Leu Leu Ala Lys Leu Gln Gly Leu Ile Pro Arg 425 430 435			1532
80	TAC CCA CTC GAC GAA CTC CTT AGA GGA TAC ACC ACT GAT CTT ATC TTT Tyr Pro Leu Asp Glu Leu Leu Arg Gly Tyr Thr Thr Asp Leu Ile Phe 440 445 450			1580
85	CCC TTA TCG ACA GAG GCA GTC CAG GCT CGG AAG ACG CCT ATC GAG ACC Pro Leu Ser Thr Glu Ala Val Gln Ala Arg Lys Thr Pro Ile Glu Thr 455 460 465			1628

ACA GCT GAC TTG CTG GAC TAT GGT CTA TGT GTC GCA GGC TCA GTC GCC	1676
Thr Ala Asp Leu Leu Asp Tyr Gly Leu Cys Val Ala Gly Ser Val Ala	
470 475 480	
5 GAG CTA TTG GTC TAT GTC TCT TGG GCA AGT GCA CCA AGT CAG GTC CCT	1724
Glu Leu Leu Val Tyr Val Ser Trp Ala Ser Ala Pro Ser Gln Val Pro	
485 490 495 500	
10 GCC ACC ATA GAA GAA AGA GAA GCT GTG TTA GTG GCA AGC CGA GAG ATG	1772
Ala Thr Ile Glu Glu Arg Glu Ala Val Leu Val Ala Ser Arg Glu Met	
505 510 515	
15 GGA ACT GCC CTT CAG TTG GTG AAC ATT GCT AGG GAC ATT AAA GGG GAC	1820
Gly Thr Ala Leu Gln Leu Val Asn Ile Ala Arg Asp Ile Lys Gly Asp	
520 525 530	
20 GCA ACA GAA GGG AGA TTT TAC CTA CCA CTC TCA TTC TTT GGT CTT CGG	1868
Ala Thr Glu Gly Arg Phe Tyr Leu Pro Leu Ser Phe Phe Gly Leu Arg	
535 540 545	
25 GAT GAA TCA AAG CTT GCG ATC CCG ACT GAT TGG ACG GAA CCT CGG CCT	1916
Asp Glu Ser Lys Leu Ala Ile Pro Thr Asp Trp Thr Glu Pro Arg Pro	
550 555 560	
30 CAA GAT TTC GAC AAA CTC CTC AGT CTA TCT CCT TCG TCC ACA TTA CCA	1964
Gln Asp Phe Asp Lys Leu Leu Ser Leu Ser Pro Ser Ser Thr Leu Pro	
565 570 575 580	
35 TCT TCA AAC GCC TCA GAA AGC TTC CGG TTC GAA TGG AAG ACG TAC TCG	2012
Ser Ser Asn Ala Ser Glu Ser Phe Arg Phe Glu Trp Lys Thr Tyr Ser	
585 590 595	
40 CTT CCA TTA GTC GCC TAC GCA GAG GAT CTT GCC AAA CAT TCT TAT AAG	2060
Leu Pro Leu Val Ala Tyr Ala Glu Asp Leu Ala Lys His Ser Tyr Lys	
600 605 610	
45 GGA ATT GAC CGA CTT CCT ACC GAG GTT CAA GCG GGA ATG CGA CGG GCT	2108
Gly Ile Asp Arg Leu Pro Thr Glu Val Gln Ala Gly Met Arg Ala Ala	
615 620 625	
50 TGC GCG AGC TAC CTA CTG ATC GGC CGA GAG ATC AAA GTC GTT TGG AAA	2156
Cys Ala Ser Tyr Leu Leu Ile Gly Arg Glu Ile Lys Val Val Trp Lys	
630 635 640	
55 GGA GAC GTC GGA GAG AGA AGG ACA GTT GCC GGA TGG AGG AGA GTC CGG	2204
Gly Asp Val Gly Glu Arg Arg Thr Val Ala Gly Trp Arg Arg Val Arg	
645 650 655 660	
60 AAA GTC TTG AGT GTG GTC ATG AGC GGA TGG GAA GGG CAG TAAGACAGCG	2253
Lys Val Leu Ser Val Val Met Ser Gly Trp Glu Gly Gln	
665 670	
65 GAAAGAATACC GACAGACAAT GATGAGTGAG AATAAAATCA TCTTCAATCT TCCTTCTCTA	2313
GGTGCTCTTT TTGTTTTCTT ATTATGACCA ACTCTAAAGG AACTGGCCCTT GCAGATATTT	2373
CTCTTCCCCC CAATCTCCCTC CTTCCTCATCG TTGTTCTTT CCATTTTGT CGGTTTACTA	2433
66 TGTCATTCTT TTGTTCTTGT TTGTTCTTATC AATCTAGACA ATTCTATAGA TGTTTGAAT	2493
TTATACATIG ACAGGTTATA GACCATAAAG ACTAAAAAAA AAAAAAAA AAA	2546

6 (2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 673 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

70

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

5 Met Thr Ala Leu Ala Tyr Tyr Gln Ile His Leu Ile Tyr Thr Leu Pro
 1 5 10 15

Ile Leu Gly Leu Leu Gly Leu Leu Thr Ser Pro Ile Leu Thr Lys Phe
 20 25 30

10 Asp Ile Tyr Lys Ile Ser Ile Leu Val Phe Ile Ala Phe Ser Ala Thr
 35 40 45

15 Thr Pro Trp Asp Ser Trp Ile Ile Arg Asn Gly Ala Trp Thr Tyr Pro
 50 55 60

Ser Ala Glu Ser Gly Gln Gly Val Phe Gly Thr Phe Leu Asp Val Pro
 65 70 75 80

20 Tyr Glu Glu Tyr Ala Phe Phe Val Ile Gln Thr Val Ile Thr Gly Leu
 85 90 95

Val Tyr Val Leu Ala Thr Arg His Leu Leu Pro Ser Leu Ala Leu Pro
 100 105 110

25 Lys Thr Arg Ser Ser Ala Leu Ser Leu Ala Leu Lys Ala Leu Ile Pro
 115 120 125

Leu Pro Ile Ile Tyr Leu Phe Thr Ala His Pro Ser Pro Ser Pro Asp
 130 135 140

30 Pro Leu Val Thr Asp His Tyr Phe Tyr Met Arg Ala Leu Ser Leu Leu
 145 150 155 160

35 Ile Thr Pro Pro Thr Met Leu Leu Ala Ala Leu Ser Gly Glu Tyr Ala
 165 170 175

Phe Asp Trp Lys Ser Gly Arg Ala Lys Ser Thr Ile Ala Ala Ile Met
 180 185 190

40 Ile Pro Thr Val Tyr Leu Ile Trp Val Asp Tyr Val Ala Val Gly Gln
 195 200 205

45 Asp Ser Trp Ser Ile Asn Asp Glu Lys Ile Val Gly Trp Arg Leu Gly
 210 215 220

Gly Val Leu Pro Ile Glu Glu Ala Met Phe Phe Leu Leu Thr Asn Leu
 225 230 235 240

50 Met Ile Val Leu Gly Leu Ser Ala Cys Asp His Thr Gln Ala Leu Tyr
 245 250 255

Leu Leu His Gly Arg Thr Ile Tyr Gly Asn Lys Lys Met Pro Ser Ser
 260 265 270

55 Phe Pro Leu Ile Thr Pro Pro Val Leu Ser Leu Phe Phe Ser Ser Arg
 275 280 285

60 Pro Tyr Ser Ser Gln Pro Lys Arg Asp Leu Glu Leu Ala Val Lys Leu
 290 295 300

Leu Glu Lys Lys Ser Arg Ser Phe Phe Val Ala Ser Ala Gly Phe Pro
 305 310 315 320

65 Ser Glu Val Arg Glu Arg Leu Val Gly Leu Tyr Ala Phe Cys Arg Val
 325 330 335

70 Thr Asp Asp Leu Ile Asp Ser Pro Glu Val Ser Ser Asn Pro His Ala
 340 345 350

Thr Ile Asp Met Val Ser Asp Phe Leu Thr Leu Leu Phe Gly Pro Pro
 355 360 365
 Leu His Pro Ser Gln Pro Asp Lys Ile Leu Ser Ser Pro Leu Leu Pro
 370 375 380
 Pro Ser His Pro Ser Arg Pro Thr Gly Met Tyr Pro Leu Pro Pro Pro
 385 390 395 400
 10 Pro Ser Leu Ser Pro Ala Glu Leu Val Gln Phe Leu Thr Glu Arg Val
 405 410 415
 Pro Val Gln Tyr His Phe Ala Phe Arg Leu Leu Ala Lys Leu Gln Gly
 420 425 430
 15 Leu Ile Pro Arg Tyr Pro Leu Asp Glu Leu Leu Arg Gly Tyr Thr Thr
 435 440 445
 Asp Leu Ile Phe Pro Leu Ser Thr Glu Ala Val Gln Ala Arg Lys Thr
 20 450 455 460
 Pro Ile Glu Thr Thr Ala Asp Leu Leu Asp Tyr Gly Leu Cys Val Ala
 465 470 475 480
 25 Gly Ser Val Ala Glu Leu Leu Val Tyr Val Ser Trp Ala Ser Ala Pro
 485 490 495
 Ser Gln Val Pro Ala Thr Ile Glu Glu Arg Glu Ala Val Leu Val Ala
 500 505 510
 30 Ser Arg Glu Met Gly Thr Ala Leu Gln Leu Val Asn Ile Ala Arg Asp
 515 520 525
 Ile Lys Gly Asp Ala Thr Glu Gly Arg Phe Tyr Leu Pro Leu Ser Phe
 530 535 540
 35 Phe Gly Leu Arg Asp Glu Ser Lys Leu Ala Ile Pro Thr Asp Trp Thr
 545 550 555 560
 40 Glu Pro Arg Pro Gln Asp Phe Asp Lys Leu Leu Ser Leu Ser Pro Ser
 565 570 575
 Ser Thr Leu Pro Ser Ser Asn Ala Ser Glu Ser Phe Arg Phe Glu Trp
 580 585 590
 45 Lys Thr Tyr Ser Leu Pro Leu Val Ala Tyr Ala Glu Asp Leu Ala Lys
 595 600 605
 His Ser Tyr Lys Gly Ile Asp Arg Leu Pro Thr Glu Val Gln Ala Gly
 610 615 620
 50 Met Arg Ala Ala Cys Ala Ser Tyr Leu Leu Ile Gly Arg Glu Ile Lys
 625 630 635 640
 55 Val Val Trp Lys Gly Asp Val Gly Glu Arg Arg Thr Val Ala Gly Trp
 645 650 655
 Arg Arg Val Arg Lys Val Leu Ser Val Val Met Ser Gly Trp Glu Gly
 660 665 670
 60 Gln

(2) INFORMATION FOR SEQ ID NO:14:

65 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1882 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
 (iii) HYPOTHETICAL: NO
 (iv) ANTI-SENSE: NO
 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: *Phaffia rhodozyma*

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 82..1212
 (D) OTHER INFORMATION: /product= "PRcrtE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGCACGAGCC AATTTAAAGT GCACTCAGCC ATAGCTAACCA CACAGAACTA CACATACATA	60
CACTCATCG GAACACATAG G ATG GAT TAC GCG AAC ATC CTC ACA GCA ATT Met Asp Tyr Ala Asn Ile Leu Thr Ala Ile	111
1 5 10	
CCA CTC GAG TTT ACT CCT CAG GAT GAT ATC GTG CTC CTT GAA CGG TAT Pro Leu Glu Phe Thr Pro Gln Asp Asp Ile Val Leu Leu Glu Pro Tyr	159
15 20 25	
CAC TAC CTA GGA AAG AAC CCT GGA AAA GAA ATT CGA TCA CAA CTC ATC His Tyr Leu Gly Lys Asn Pro Gly Lys Glu Ile Arg Ser Gln Leu Ile	207
30 35 40	
GAG GCT TTC AAC TAT TGG TTG GAT GTC AAG AAG GAG GAT CTC GAG GTC Glu Ala Phe Asn Tyr Trp Leu Asp Val Lys Glu Asp Leu Glu Val	255
45 50 55	
ATC CAG AAC GTT GTT GGC ATG CTA CAT ACC GCT AGC TTA TTA ATG GAC Ile Gln Asn Val Val Gly Met Leu His Thr Ala Ser Leu Leu Met Asp	303
60 65 70	
GAT GTG GAG GAT TCA TCG GTC CTC AGG CGT GGG TCG CCT GTG GCC CAT Asp Val Glu Asp Ser Ser Val Leu Arg Arg Gly Ser Pro Val Ala His	351
75 80 85 90	
CTA ATT TAC GGG ATT CCG CAG ACA ATA AAC ACT GCA AAC TAC GTC TAC Leu Ile Tyr Gly Ile Pro Gin Thr Ile Asn Thr Ala Asn Tyr Val Tyr	399
95 100 105	
TTT CTG GCT TAT CAA GAG ATC TTC AAG CCT CGC CCA ACA CGG ATA CCC Phe Leu Ala Tyr Gln Glu Ile Phe Lys Leu Arg Pro Thr Pro Ile Pro	447
110 115 120	
ATG CCT GTC ATT CCT CCT TCA TCT GCT TCG CCT CAA TCA TCC GTC TCC Met Pro Val Ile Pro Pro Ser Ser Ala Ser Leu Gln Ser Ser Val Ser	495
125 130 135	
TCT GCA TCC TCC TCC TCG GCC TCG TCT GAA AAC CGG GGC ACG TCA Ser Ala Ser Ser Ser Ala Ser Ser Glu Asn Gly Gly Thr Ser	543
140 145 150	
ACT CCT AAT TCG CAG ATT CCG TTC TCG AAA GAT ACG TAT CTT GAT AAA Thr Pro Asn Ser Gln Ile Pro Phe Ser Lys Asp Thr Tyr Leu Asp Lys	591
155 160 165 170	
GTG ATC ACA GAC GAG ATG CTT CTC CAT AGA GGG CAA GGC CTG GAG Val Ile Thr Asp Glu Met Leu Ser Leu His Arg Gly Gln Gly Leu Glu	639
175 180 185	
CTA TTC TGG AGA GAT AGT CTG ACG TGT CCT AGC GAA GAG GAA TAT GTG Leu Phe Trp Arg Asp Ser Leu Thr Cys Pro Ser Glu Glu Glu Tyr Val	687
190 195 200	

AAA ATG GTT CTT GGA AAG ACG GGA GGT TTG TTC CGT ATA CGG GTC AGA Lys Met Val Leu Gly Lys Thr Gly Gly Leu Phe Arg Ile Ala Val Arg 205 210 215	735
5 TTG ATG ATG GCA AAG TCA GAA TGT GAC ATA GAC TTT GTC CAG CTT GTC Leu Met Met Ala Lys Ser Glu Cys Asp Ile Asp Phe Val Gln Leu Val 220 225 230	783
10 AAC TTG ATC TCA ATA TAC TTC CAG ATC AGG GAT GAC TAT ATG AAC CTT Asn Leu Ile Ser Ile Tyr Phe Gln Ile Arg Asp Asp Tyr Met Asn Leu 235 240 245 250	831
15 CAG TCT TCT GAG TAT GCC CAT AAT AAG AAT TTT GCA GAG GAC CTC ACA Gln Ser Ser Glu Tyr Ala His Asn Lys Asn Phe Ala Glu Asp Leu Thr 255 260 265	879
20 GAA GGG AAA TTC AGT TTT CCC ACT ATC CAC TCG ATT CAT GCC AAC CCC Glu Gly Lys Phe Ser Phe Pro Thr Ile His Ser Ile His Ala Asn Pro 270 275 280 ..	927
25 TCA TCG AGA CTC GTC ATC AAT ACG TTG CAG AAG AAA TUG ACC TCT CCT Ser Ser Arg Leu Val Ile Asn Thr Leu Gln Lys Lys Ser Thr Ser Pro 285 290 295	975
30 GAG ATC CTT CAC CAC TGT GTC AAC TAC ATG CGC ACA GAA ACC CAC TCA Glu Ile Leu His His Cys Val Asn Tyr Met Arg Thr Glu Thr His Ser 300 305 310	1023
35 TTC GAA TAT ACT CAG GAA GTC CTC AAC ACC TTG TCA GGT GCA CTC GAG Phe Glu Tyr Thr Gln Glu Val Leu Asn Thr Leu Ser Gly Ala Leu Glu 315 320 325 330	1071
40 AGA GAA CTA GGA AGG CTT CAA GGA GAG TTC GCA GAA GCT AAC TCA AGG Arg Glu Leu Gly Arg Leu Gln Gly Glu Phe Ala Glu Ala Asn Ser Arg 335 340 345	1119
45 ATG GAT CTT GGA GAC GTC GAT TCG GAA GGA AGA ACG GGG AAG AAC GTC Met Asp Leu Gly Asp Val Asp Ser Glu Gly Arg Thr Gly Lys Asn Val 350 355 360	1167
50 AAA TTG GAA GCG ATC CTG AAA AAG CTA GCC GAT ATC CCT CTG TGAAAGAACAA Lys Leu Glu Ala Ile Leu Lys Lys Leu Ala Asp Ile Pro Leu 365 370 375	1219
55 TATTCCTCTCT CTOGTCCTGTC CGTTTCTATTC AGGGTTTTAT AAGTTGTCCTC TTATTCCTTA AGGGTTTGTC AGATGATGCG ACTTGTATGTG CTCTATGTC CGTTCATCTT TTTCACTTGG 60 ACTTTTTCTT CTACCGTGCA TGCCCCATTGG CATTCTCTTG TTTCATCTTGT GTTAAATTGG TTGGACATAA CATTAAATCAT CGTGCTCTCT TCTTTTUGAA GAAATCTGT GACTTGTTGA ACITCAACTA TAATTAATCA TATTCAATTC TCAAAGTCCT OGTCCTCTGG CAATGTGATT 65 CCTCCCTTCCA GTCCCCCTT TGATTTCCCT CTCTTGGATC GGTTTCTTTT TCCTTTTTCG TCTCCCTGCT CTCTTTTATT CGCTTTCGGT CTCTCTGACT CGTTTCTCT TCACTTTTT 70 TTTTCATCTT CTCTGGTCA ACTTGTCAATT TAATCTCTCT AGGGTCCTAT GTCAACACGT GCCAAGCATG TCATACGTGT GCAGGGTGAT GTACAGTCAT TTGCCCCATCC CTCTTGGCAG GGTCCTCATCT ATCTTGTCCTA TCGACTTTTC CTCTTTTGA ATTTCCTGG AGTTTTATCT 75 TGGTATAAGC AATGGAGAAG AGGGCAAAA AAAAAAAA AAAAAAAA AAAAAACTCG AGG	1279 1339 1399 1459 1519 1579 1639 1699 1759 1819 1879 1882

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 376 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Met Asp Tyr Ala Asn Ile Leu Thr Ala Ile Pro Leu Glu Phe Thr Pro
 1 5 10 15

15 Gln Asp Asp Ile Val Leu Leu Glu Pro Tyr His Tyr Leu Gly Lys Asn
 20 25 30

Pro Gly Lys Glu Ile Arg Ser Gln Leu Ile Glu Ala Phe Asn Tyr Trp
 35 40 45

20 Leu Asp Val Lys Lys Glu Asp Leu Glu Val Ile Gln Asn Val Val Gly
 50 55 60

Met Leu His Thr Ala Ser Leu Leu Met Asp Asp Val Glu Asp Ser Ser
 25 65 70 75 80

Val Leu Arg Arg Gly Ser Pro Val Ala His Leu Ile Tyr Gly Ile Pro
 85 90 95

30 Gln Thr Ile Asn Thr Ala Asn Tyr Val Tyr Phe Leu Ala Tyr Gln Glu
 100 105 110

Ile Phe Lys Leu Arg Pro Thr Pro Ile Pro Met Pro Val Ile Pro Pro
 115 120 125

35 Ser Ser Ala Ser Leu Gln Ser Ser Val Ser Ser Ala Ser Ser Ser
 130 135 140

40 Ser Ala Ser Ser Glu Asn Gly Gly Thr Ser Thr Pro Asn Ser Gln Ile
 145 150 155 160

Pro Phe Ser Lys Asp Thr Tyr Leu Asp Lys Val Ile Thr Asp Glu Met
 165 170 175

45 Leu Ser Leu His Arg Gly Gln Gly Leu Glu Leu Phe Trp Arg Asp Ser
 180 185 190

Leu Thr Cys Pro Ser Glu Glu Tyr Val Lys Met Val Leu Gly Lys
 50 195 200 205

Thr Gly Gly Leu Phe Arg Ile Ala Val Arg Leu Met Met Ala Lys Ser
 210 215 220

55 Glu Cys Asp Ile Asp Phe Val Gln Leu Val Asn Leu Ile Ser Ile Tyr
 225 230 235 240

Phe Gln Ile Arg Asp Asp Tyr Met Asn Leu Gln Ser Ser Glu Tyr Ala
 245 250 255

60 His Asn Lys Asn Phe Ala Glu Asp Leu Thr Glu Gly Lys Phe Ser Phe
 260 265 270

Pro Thr Ile His Ser Ile His Ala Asn Pro Ser Ser Arg Leu Val Ile
 65 275 280 285

Asn Thr Leu Gln Lys Lys Ser Thr Ser Pro Glu Ile Leu His His Cys
 290 295 300

70 Val Asn Tyr Met Arg Thr Glu Thr His Ser Phe Glu Tyr Thr Gln Glu

305	310	315	320
Val Leu Asn Thr Leu Ser Gly Ala Leu Glu Arg Glu Leu Gly Arg Leu			
325	330	335	
Gln Gly Glu Phe Ala Glu Ala Asn Ser Arg Met Asp Leu Gly Asp Val			
340	345	350	
Asp Ser Glu Gly Arg Thr Gly Lys Asn Val Lys Leu Glu Ala Ile Leu			
355	360	365	
Lys Lys Leu Ala Asp Ile Pro Leu			
370	375		

15 (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2058 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- 10 (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: *Phaffia rhodozyma*
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 46..1794
 - (D) OTHER INFORMATION: /product= "PRcrtI"

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CCTCGCCGAA TCTAACTTGA CACATAACTC TAGTATCTAT ACTCG ATG GGA AAA	54
Met Gly Lys	
1	
45 GAA CAA GAT CAG GAT AAA CCC ACA GCT ATC ATC GIG GGA TGT GGT ATC	102
Glu Gln Asp Gln Asp Lys Pro Thr Ala Ile Ile Val Gly Cys Gly Ile	
5 10 15	
50 GGT GGA ATC GCC ACT GCC GCT CGT CTT GCT AAA GAA GGT TTC CAG GTC	150
Gly Gly Ile Ala Thr Ala Ala Arg Leu Ala Lys Glu Gly Phe Gln Val	
20 25 30 35	
55 ACG GTG TTC GAG AAG AAC GAC TAC TCC GGA GGT CGA TGC TCT TTA ATC	198
Thr Val Phe Glu Lys Asn Asp Tyr Ser Gly Gly Arg Cys Ser Leu Ile	
40 45 50	
60 GAG CGA GAT GGT TAT CGA TTC GAT CAG GGG CCC AGT TTG CTG CTC TTG	246
Glu Arg Asp Gly Tyr Arg Phe Asp Gln Gly Pro Ser Leu Leu Leu	
55 60 65	
65 CCA GAT CTC TTC AAG CAG ACA TTC GAA GAT TTG GGA GAG AAG ATG GAA	294
Pro Asp Leu Phe Lys Gln Thr Phe Glu Asp Leu Gly Glu Lys Met Glu	
70 75 80	
70 GAT TGG GTC GAT CTC ATC AAG TGT GAA CCC AAC TAT GTT TGC CAC TTC	342
Asp Trp Val Asp Leu Ile Lys Cys Glu Pro Asn Tyr Val Cys His Phe	
85 90 95	
75 CAC GAT GAA GAG ACT TTC ACT TTT TCA ACC GAC ATG GCG TTG CTC AAG	390
His Asp Glu Glu Thr Phe Thr Phe Ser Thr Asp Met Ala Leu Leu Lys	

100	105	110	115	
CGG GAA GTC GAG CGT TTT GAA GGC AAA GAT GGA TTT GAT CGG TTC TTG Arg Glu Val Glu Arg Phe Glu Gly Lys Asp Gly Phe Asp Arg Phe Leu 120 125 130				438
TCG TTT ATC CAA GAA GCC CAC AGA CAT TAC GAG CTT GCT GTC GTT CAC Ser Phe Ile Gln Glu Ala His Arg His Tyr Glu Leu Ala Val Val His 135 140 145				486
10 GTC CTG CAG AAG AAC TTC CCT GGC TTC GCA GCA TTC TTA CGG CTA CAG Val Leu Gln Lys Asn Phe Pro Gly Phe Ala Ala Phe Leu Arg Leu Gln 150 155 160				534
15 TTC ATT GGC CAA ATC CTG GCT CTT CAC CCC TTC GAG TCT ATC TGG ACA Phe Ile Gly Gln Ile Leu Ala Leu His Pro Phe Glu Ser Ile Trp Thr 165 170 175				582
20 AGA GTT TGT CGA TAT TTC AAG ACC GAC AGA TTA CGA AGA GTC TTC TCG Arg Val Cys Arg Tyr Phe Lys Thr Asp Arg Leu Arg Arg Val Phe Ser 180 185 190 195				630
25 TTT GCA GTG ATG TAC ATG GGT CAA AGC CCA TAC AGT GCG CCC GGA ACA Phe Ala Val Met Tyr Met Gly Gln Ser Pro Tyr Ser Ala Pro Gly Thr 200 205 210				678
30 TAT TCC TTG CTC CAA TAC ACC GAA TTG ACC GAG GGC ATC TGG TAT CGG Tyr Ser Leu Leu Gln Tyr Thr Glu Leu Thr Glu Gly Ile Trp Tyr Pro 215 220 225				726
35 AGA GGA GGC TTT TGG CAG GTT CCT AAT ACT CTT CTT CAG ATC GTC AAG Arg Gly Phe Trp Gln Val Pro Asn Thr Leu Leu Gln Ile Val Lys 230 235 240				774
40 CGC AAC AAT CCC TCA GCC AAG TTC AAT TTC AAC GCT CCA GTT TCC CAG Arg Asn Asn Pro Ser Ala Lys Phe Asn Phe Asn Ala Pro Val Ser Gln 245 250 255				822
45 GTT CTT CTC TCT CCT GCC AAG GAC CGA GCG ACT GGT GTT CGA CTT GAA Val Leu Leu Ser Pro Ala Lys Asp Arg Ala Thr Gly Val Arg Leu Glu 260 265 270 275				870
50 TCC GGC GAG GAA CAT CAC GCC GAT GTT GTG ATT GTC AAT GCT GAC CTC Ser Gly Glu Glu His His Ala Asp Val Val Ile Val Asn Ala Asp Leu 280 285 290				918
55 GTT TAC GCC TCC GAG CAC TTG ATT CCT GAC GAT GCC AGA AAC AAG ATT Val Tyr Ala Ser Glu His Leu Ile Pro Asp Asp Ala Arg Asn Lys Ile 295 300 305				966
60 GGC CAA CTG GGT GAA GTC AAG AGA AGT TGG TGG GCT GAC TTA GTT GGT Gly Gln Leu Gly Glu Val Lys Arg Ser Trp Trp Ala Asp Leu Val Gly 310 315 320				1014
65 GGA AAG AAG CTC AAG GGA AGT TGC AGT AGT TTG AGC TTC TAC TGG AGC Gly Lys Lys Leu Lys Gly Ser Cys Ser Ser Leu Ser Phe Tyr Trp Ser 325 330 335				1062
70 ATG GAC CGA ATC GTG GAC GGT CTG GGC GGA CAC AAT ATC TTC TTG GCC Met Asp Arg Ile Val Asp Gly Leu Gly His Asn Ile Phe Leu Ala 340 345 350 355				1110
GAG GAC TTC AAG GGA TCA TTC GAC ACA ATC TTC GAG GAG TTG GGT CTC Glu Asp Phe Lys Gly Ser Phe Asp Thr Ile Phe Glu Glu Leu Gly Leu 360 365 370				1158
CCA GCC GAT CCT TCC TTT TAC GTG AAC GTT CCC TCG CGA ATC GAT CCT Pro Ala Asp Pro Ser Phe Tyr Val Asn Val Pro Ser Arg Ile Asp Pro 375 380 385				1206

1254	TCT GCC GCT CCC GAA GGC AAA GAT GCT ATC GTC ATT CTT GTG CCG TGT Ser Ala Ala Pro Glu Gly Lys Asp Ala Ile Val Ile Leu Val Pro Cys 390 395 400
1302	5 GGC CAT ATC GAC GCT TCG AAC CCT CAA GAT TAC AAC AAG CTT GTT GCT Gly His Ile Asp Ala Ser Asn Pro Gln Asp Tyr Asn Lys Leu Val Ala 405 410 415
1350	10 CGG GCA AGG AAG TTT GTG ATC CAA ACG CTT TCC GCC AAG CTT GGA CTT Arg Ala Arg Lys Phe Val Ile Gln Thr Leu Ser Ala Lys Leu Gly Leu 420 425 430 435
1398	15 CCC GAC TTT GAA AAA ATG ATT GTG GCA GAG AAG GTT CAC GAT GCT CCC Pro Asp Phe Glu Lys Met Ile Val Ala Glu Lys Val His Asp Ala Pro 440 445 450
1446	20 TCT TGG GAG AAA GAA TTT AAC CTC AAG GAC GGA AGC ATC TTG GGA CTG Ser Trp Glu Lys Glu Phe Asn Leu Lys Asp Gly Ser Ile Leu Gly Leu 455 460 465
1494	25 GCT CAC AAC TTT ATG CAA GTT CTT GGT TTC AGG CCG AGC ACC AGA CAT Ala His Asn Phe Met Gln Val Leu Gly Phe Arg Pro Ser Thr Arg His 470 475 480
1542	30 CCC AAG TAT GAC AAG TTG TTC TTT GTC GGG GCT TCG ACT CAT CCC GGA Pro Lys Tyr Asp Lys Leu Phe Val Gly Ala Ser Thr His Pro Gly 485 490 495
1590	35 ACT GGG GTT CCC ATC GTC TTG GCT GGA GCC AAG TTA ACT GCC AAC CAA Thr Gly Val Pro Ile Val Leu Ala Gly Ala Lys Leu Thr Ala Asn Gln 500 505 510 515
1638	40 GTT CTC GAA TCC TTT GAC CGA TCC CCA GCT CCA GAT CCC AAT ATG TCA Val Leu Glu Ser Phe Asp Arg Ser Pro Ala Pro Asp Pro Asn Met Ser 520 525 530
1686	45 CTC TCC GTA CCA TAT GGA AAA CCT CTC AAA TCA AAT GGA ACG GGT ATC Leu Ser Val Pro Tyr Gly Lys Pro Leu Lys Ser Asn Gly Thr Gly Ile 535 540 545
1734	50 GAT TCT CAG GTC CAG CTG AAG TTC ATG GAT TTG GAG AGA TGG GTC TAC Asp Ser Gln Val Gln Leu Lys Phe Met Asp Leu Glu Arg Trp Val Tyr 550 555 560
1782	55 CTT TTG GTG TTG ATT GGG GCC GTG ATC GCT CGA TCC GTT GGT GTT Leu Leu Val Leu Leu Ile Gly Ala Val Ile Ala Arg Ser Val Gly Val 565 570 575
1831	60 CTT GCT TTC TGAAGCAAGA CAACGATCGT TCTCTAGAGT TTTTTTTAGT Leu Ala Phe 580
1891	65 CTCTTCTCTGT GTTCTCTCTA TATACATACT CTGCTGCTCT GTTCTCTCT CGAGGGTTCCT
1951	70 TCTTCTACTTT GTGTCAGAGT CATAACGGT CTCTCTAAC GTCGTTTGA GGGCTAGACA
2011	75 ATTTGTTAGTC TCGAAATCTC CATCACCTCA AGTCTGATGT TCATCATCTT TTTTATTCGT
2058	80 TGCAATATAC ATGACTGTTA TGGACCGAAA AAAAAAAA AAAAAAA

(2) INFORMATION FOR SEQ ID NO:17:

65 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 582 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

70 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Gly Lys Glu Gln Asp Gln Asp Lys Pro Thr Ala Ile Ile Val Gly
 1 5 10 15

5 Cys Gly Ile Gly Gly Ile Ala Thr Ala Ala Arg Leu Ala Lys Glu Gly
 20 25 30

10 Phe Gln Val Thr Val Phe Glu Lys Asn Asp Tyr Ser Gly Gly Arg Cys
 35 40 45

Ser Leu Ile Glu Arg Asp Gly Tyr Arg Phe Asp Gln Gly Pro Ser Leu
 50 55 60

15 Leu Leu Leu Pro Asp Leu Phe Lys Gln Thr Phe Glu Asp Leu Gly Glu
 65 70 75 80

Lys Met Glu Asp Trp Val Asp Leu Ile Lys Cys Glu Pro Asn Tyr Val
 85 90 95

20 Cys His Phe His Asp Glu Glu Thr Phe Thr Phe Ser Thr Asp Met Ala
 100 105 110

25 Leu Leu Lys Arg Glu Val Glu Arg Phe Glu Gly Lys Asp Gly Phe Asp
 115 120 125

Arg Phe Leu Ser Phe Ile Gln Glu Ala His Arg His Tyr Glu Leu Ala
 130 135 140

30 Val Val His Val Leu Gln Lys Asn Phe Pro Gly Phe Ala Ala Phe Leu
 145 150 155 160

Arg Leu Gln Phe Ile Gly Gln Ile Leu Ala Leu His Pro Phe Glu Ser
 165 170 175

35 Ile Trp Thr Arg Val Cys Arg Tyr Phe Lys Thr Asp Arg Leu Arg Arg
 180 185 190

40 Val Phe Ser Phe Ala Val Met Tyr Met Gly Gln Ser Pro Tyr Ser Ala
 195 200 205

Pro Gly Thr Tyr Ser Leu Leu Gln Tyr Thr Glu Leu Thr Glu Gly Ile
 210 215 220

45 Trp Tyr Pro Arg Gly Gly Phe Trp Gln Val Pro Asn Thr Leu Leu Gln
 225 230 235 240

Ile Val Lys Arg Asn Asn Pro Ser Ala Lys Phe Asn Phe Asn Ala Pro
 245 250 255

50 Val Ser Gln Val Leu Leu Ser Pro Ala Lys Asp Arg Ala Thr Gly Val
 260 265 270

Arg Leu Glu Ser Gly Glu Glu His His Ala Asp Val Val Ile Val Asn
 275 280 285

Ala Asp Leu Val Tyr Ala Ser Glu His Leu Ile Pro Asp Asp Ala Arg
 290 295 300

60 Asn Lys Ile Gly Gln Leu Gly Glu Val Lys Arg Ser Trp Trp Ala Asp
 305 310 315 320

Leu Val Gly Gly Lys Lys Leu Lys Gly Ser Cys Ser Ser Leu Ser Phe
 325 330 335

65 Tyr Trp Ser Met Asp Arg Ile Val Asp Gly Leu Gly Gly His Asn Ile
 340 345 350

70 Phe Leu Ala Glu Asp Phe Lys Gly Ser Phe Asp Thr Ile Phe Glu Glu
 355 360 365

45 (2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2470 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: do

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(int) ANSEL-SENNE: NO

(iii) ORIGINAL SOURCE:

60 (A) ORGANISM: *Phaffia rhodozyma*

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 177..2198

65 (D) OTHER INFORMATION: /product= "PRcrTY"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AACAAGAAGT GGACACAGAG AGATCTTTCG TGAAGAGTTG TATTOCAGAA AGGGAAAAACA

10	AAGGAAAGAA GCGCCGAAGC ACATCACCAA CTTCAGCAAG CGGGTCCAGC CGGATCTCGG ATAGACATCA TCTTACCCAA CTGTTATCAT CCCAACAGA TAGAGTTTTT GTCGCA	120 176
15	ATG ACG GCT CTC GCA TAT TAC CAG ATC CAT CTG ATC TAT ACT CTC CCA Met Thr Ala Leu Ala Tyr Tyr Gln Ile His Leu Ile Tyr Thr Leu Pro 1 5 10 15	224
20	ATT CTT GGT CTT CTC GGC CTG CTC ACT TCC CGG ATT TTG ACA AAA TTT Ile Leu Gly Leu Leu Gly Leu Leu Thr Ser Pro Ile Leu Thr Lys Phe 20 25 30	272
25	GAC ATC TAC AAA ATA TCG ATC CTC GTA TTT ATT GCG TTT AGT GCA ACC Asp Ile Tyr Lys Ile Ser Ile Leu Val Phe Ile Ala Phe Ser Ala Thr 35 40 45	320
30	ACA CCA TGG GAC TCA TGG ATC ATC AGA AAT GGC GCA TGG ACA TAT CCA Thr Pro Trp Asp Ser Trp Ile Ile Arg Asn Gly Ala Trp Thr Tyr Pro 50 55 60	368
35	TCA GCG GAG AGT GGC CAA GGC GTG TTT GGA ACG TTT CTA GAT GTT CCA Ser Ala Glu Ser Gly Gln Gly Val Phe Gly Thr Phe Leu Asp Val Pro 65 70 75 80	416
40	TAT GAA GAG TAC GCT TTC TTG GTC ATT CAA ACC GTC ATC ACC GGC TTG Tyr Glu Glu Tyr Ala Phe Phe Val Ile Gln Thr Val Ile Thr Gly Leu 85 90 95	464
45	GTC TAC GTC TTG GCA ACT AGG CAC CTT CTC CCA TCT CTC GGG CTT CCC Val Tyr Val Ala Thr Arg His Leu Leu Pro Ser Leu Ala Leu Pro 100 105 110	512
50	AAG ACT AGA TCG TCC GCC CTT TCT CTC GGG CTC AAG GGG CTC ATC CCT Lys Thr Arg Ser Ser Ala Leu Ser Leu Ala Leu Lys Ala Leu Ile Pro 115 120 125	560
55	CTG CCC ATT ATC TAC CTA TTT ACC GCT CAC CCC AGC CCA TCG CCC GAC Leu Pro Ile Ile Tyr Leu Phe Thr Ala His Pro Ser Pro Ser Pro Asp 130 135 140	608
60	CCG CTC GTG ACA GAT CAC TAC TTC TAC ATG CGG GCA CTC TCC TTA CTC Pro Leu Val Thr Asp His Tyr Phe Tyr Met Arg Ala Leu Ser Leu Leu 145 150 155 160	656
65	ATC ACC CCA CCT ACC ATG CTC TTG GCA GCA TTA TCA GGC GAA TAT GCT Ile Thr Pro Pro Thr Met Leu Leu Ala Ala Leu Ser Gly Glu Tyr Ala 165 170 175	704
70	TTC GAT TGG AAA AGT GGC CGA GCA AAG TCA ACT ATT GCA GCA ATC ATG Phe Asp Trp Lys Ser Gly Arg Ala Lys Ser Thr Ile Ala Ala Ile Met 180 185 190	752
75	ATC CCG AGC GTG TAT CTG ATT TGG GTC GAT TAT GTT GCT GTC GGT CAA Ile Pro Thr Val Tyr Leu Ile Trp Val Asp Tyr Val Ala Val Gly Gln 195 200 205	800
80	GAC TCT TGG TCG ATC AAC GAT GAG AAG ATT GTC GGG TGG AGG CTT GGA Asp Ser Trp Ser Ile Asn Asp Glu Lys Ile Val Gly Trp Arg Leu Gly 210 215 220	848
85	GGT GTC CTC CCC ATT GAG GAA GCT ATG TTC TTC TTA CTG ACG AAT CTC Gly Val Leu Pro Ile Glu Ala Met Phe Phe Leu Leu Thr Asn Leu 225 230 235 240	896
90	ATG ATT GTT CTG GGT CTG TCT GCC TGC GAT CAT ACT CAG GGC CTC TAC Met Ile Val Leu Gly Leu Ser Ala Cys Asp His Thr Gln Ala Leu Tyr 245 250 255	944
95	CTG CTC CAC GGT CGA ACT ATT TAT GGC AAC AAA AAG ATG CCA TCT TCA Leu Leu His Gly Arg Thr Ile Tyr Gly Asn Lys Lys Met Pro Ser Ser	992

	260	265	270	
5	TTT CCC CTC ATT ACA CGA CCT GTG CTC TCC CTG TTT TTT AGC AGC CGA Phe Pro Leu Ile Thr Pro Pro Val Leu Ser Leu Phe Phe Ser Ser Arg 275 280 285			1040
10	CCA TAC TCT TCT CAG CCA AAA CGT GAC TTG GAA CTG GCA GTC AAG TTG Pro Tyr Ser Ser Gln Pro Lys Arg Asp Leu Glu Leu Ala Val Lys Leu 290 295 300			1088
15	TTG GAG AAA AAG AGC CGG AGC TTT TTT GTT GCC TCG GCT GGA TTT CCT Leu Glu Lys Lys Ser Arg Ser Phe Phe Val Ala Ser Ala Gly Phe Pro 305 310 315 320			1136
20	AGC GAA GTT AGG GAG AGG CTG GTT GGA CTA TAC GCA TTC TGC CGG GTG Ser Glu Val Arg Glu Arg Leu Val Gly Leu Tyr Ala Phe Cys Arg Val 325 330 335			1184
25	ACT GAT GAT CTT ATC GAC TCT CCT GAA GAA TCT TCC AAC CGG CAT GCC Thr Asp Asp Leu Ile Asp Ser Pro Glu Val Ser Ser Asn Pro His Ala 340 345 350			1232
30	ACA ATT GAC ATG GTC TCC GAT TTT CTT ACC CTA CTA TTT GGG CCC CGG Thr Ile Asp Met Val Ser Asp Phe Leu Thr Leu Leu Phe Gly Pro Pro 355 360 365			1280
35	CTA CAC CCT TCG CAA CCT GAC AAG ATC CTT TCT TCG CCT TTA CTT CCT Leu His Pro Ser Gln Pro Asp Lys Ile Leu Ser Ser Pro Leu Leu Pro 370 375 380			1328
40	CCT TCG CAC CCT TCC CGA CCC ACG GGA ATG TAT CCC CTC CGG CCT CCT Pro Ser His Pro Ser Arg Pro Thr Gly Met Tyr Pro Leu Pro Pro Pro 385 390 395 400			1376
45	CCT TCG CTC TCG CCT GCC GAG CTC GTT CAA TTC CTT ACC GAA AGG GTT Pro Ser Leu Ser Pro Ala Glu Leu Val Gln Phe Leu Thr Glu Arg Val 405 410 415			1424
50	CCC GTT CAA TAC CAT TTC GCC TTC AGG TTG CTC GCT AAG TTG CAA GGG Pro Val Gln Tyr His Phe Ala Phe Arg Leu Leu Ala Lys Leu Gln Gly 420 425 430			1472
55	CTG ATC CCT CGA TAC CCA CTC GAC GAA CTC CTT AGA GGA TAC ACC ACT Leu Ile Pro Arg Tyr Pro Leu Asp Glu Leu Leu Arg Gly Tyr Thr Thr 435 440 445			1520
60	GAT CTT ATC TTT CCC TTA TCG ACA GAG GCA GTC CAG GCT CGG AAG ACG Asp Leu Ile Phe Pro Leu Ser Thr Glu Ala Val Gln Ala Arg Lys Thr 450 455 460			1568
65	CCT ATC GAG ACC ACA GCT GAC TTG CTC GAC TAT GGT CTA TGT GTC GCA Pro Ile Glu Thr Thr Ala Asp Leu Leu Asp Tyr Gly Leu Cys Val Ala 465 470 475 480			1616
70	GGC TCA GTC GCC GAG CTA TTG GTC TAT GTC TCT TGG GCA AGT GCA CCA Gly Ser Val Ala Glu Leu Leu Val Tyr Val Ser Trp Ala Ser Ala Pro 485 490 495			1664
75	AGT CAG GTC CCT GCC ACC ATA GAA GAA AGA GAA GCT GTG TTA GTG GCA Ser Gln Val Pro Ala Thr Ile Glu Glu Arg Glu Ala Val Leu Val Ala 500 505 510			1712
80	AGC CGA GAG ATG GGA ACT GCC CTT CAG TTG GTG AAC ATT GCT AGG GAC Ser Arg Glu Met Gly Thr Ala Leu Gln Leu Val Asn Ile Ala Arg Asp 515 520 525			1760
85	ATT AAA GGG GAC GCA ACA GAA GGG AGA TTT TAC CTA CCA CTC TCA TTC Ile Lys Gly Asp Ala Thr Glu Gly Arg Phe Tyr Leu Pro Leu Ser Phe 530 535 540			1808

5	TTT GGT CTT CGG GAT GAA TCA AAG CTT GCG ATC CCG ACT GAT TGG ACG Phe Gly Leu Arg Asp Glu Ser Lys Leu Ala Ile Pro Thr Asp Trp Thr 545 550 555 560	1856
5	GAA CCT CGG CCT CAA GAT TTC GAC AAA CTC CTC AGT CTA TCT CCT TCG Glu Pro Arg Pro Gln Asp Phe Asp Lys Leu Leu Ser Leu Ser Pro Ser 565 570 575	1904
10	TCC ACA TTA CCA TCT TCA AAC GCC TCA GAA AGC TTC CGG TTC GAA TGG Ser Thr Leu Pro Ser Ser Asn Ala Ser Glu Ser Phe Arg Phe Glu Trp 580 585 590	1952
15	AAG ACG TAC TCG CTT CCA TTA GTC GCC TAC GCA GAG GAT CTT GCC AAA Lys Thr Tyr Ser Leu Pro Leu Val Ala Tyr Ala Glu Asp Leu Ala Lys 595 600 605	2000
20	CAT TCT TAT AAG GGA ATT GAC CGA CTT CCT ACC GAG GTT CAA GCG GGA His Ser Tyr Lys Gly Ile Asp Arg Leu Pro Thr Glu Val Gln Ala Gly 610 615 620	2048
25	ATG CGA GCG GCT TGC GCG AGC TAC CTA CTG ATC GGC CGA GAG ATC AAA Met Arg Ala Ala Cys Ala Ser Tyr Leu Leu Ile Gly Arg Glu Ile Lys 625 630 635 640	2096
25	GTC GTT TGG AAA GGA GAC GTC GGA GAG AGA AGG ACA GTT GCC GGA TGG Val Val Trp Lys Gly Asp Val Gly Glu Arg Arg Thr Val Ala Gly Trp 645 650 655	2144
30	AGG AGA GTA CGG AAA GTC TTG AGT GTG GTC ATG AGC GGA TGG GAA GGG Arg Arg Val Arg Lys Val Leu Ser Val Val Met Ser Gly Trp Glu Gly 660 665 670	2192
35	CAG TAAGACAGCG GAAGAAATACC GACAGACAAT GATGAGTGAG AATAAAAATCA Gln	2245
40	TCTCTAAATCT TCTTTCTCTA GGIGCTCTTT TTIGTTTCT ATTATGACCA ACTCTAAAGG AACTGGCCCTT GCAGATATTT CTCTTCCCCC CATCTTCCCTC CTTTCCATCG TTGTTCTTT CCATTTTGT CGGTTTACTA TGTCATTCCT TTTCTTATAC AATCTAGACA ATTCTATAGA TGTTTAGAAT TTATACAAAAA AAAAAA AAAAAA	2305 2365 2425 2470

(2) INFORMATION FOR SEQ ID NO:19:

50 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 673 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: protein

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Met	Thr	Ala	Leu	Ala	Tyr	Tyr	Gln	Ile	His	Leu	Ile	Tyr	Thr	Leu	Pro
1					5					10					15
60															
Ile	Leu	Gly	Leu	Leu	Gly	Leu	Leu	Thr	Ser	Pro	Ile	Leu	Thr	Lys	Phe
								20		25				30	
65															
Asp	Ile	Tyr	Lys	Ile	Ser	Ile	Leu	Val	Phe	Ile	Ala	Phe	Ser	Ala	Thr
								35		40			45		
70															
Thr	Pro	Trp	Asp	Ser	Trp	Ile	Ile	Arg	Asn	Gly	Ala	Trp	Thr	Tyr	Pro
								50		55			60		
75															
Ser	Ala	Glu	Ser	Gly	Gln	Gly	Val	Phe	Gly	Thr	Phe	Leu	Asp	Val	Pro

65	70	75	80
Tyr Glu Glu Tyr Ala Phe Phe Val Ile Gln Thr Val Ile Thr Gly Leu			
85	90	95	
5 Val Tyr Val Leu Ala Thr Arg His Leu Leu Pro Ser Leu Ala Leu Pro			
100	105	110	
10 Lys Thr Arg Ser Ser Ala Leu Ser Leu Ala Leu Lys Ala Leu Ile Pro			
115	120	125	
15 Leu Pro Ile Ile Tyr Leu Phe Thr Ala His Pro Ser Pro Ser Pro Asp			
130	135	140	
19 Pro Leu Val Thr Asp His Tyr Phe Tyr Met Arg Ala Leu Ser Leu Leu			
145	150	155	160
20 Ile Thr Pro Pro Thr Met Leu Leu Ala Ala Leu Ser Gly Glu Tyr Ala			
165	170	175	
25 Phe Asp Trp Lys Ser Gly Arg Ala Lys Ser Thr Ile Ala Ala Ile Met			
180	185	190	
30 Ile Pro Thr Val Tyr Leu Ile Trp Val Asp Tyr Val Ala Val Gly Gln			
195	200	205	
35 Asp Ser Trp Ser Ile Asn Asp Glu Lys Ile Val Gly Trp Arg Leu Gly			
210	215	220	
40 Gly Val Leu Pro Ile Glu Glu Ala Met Phe Phe Leu Leu Thr Asn Leu			
225	230	235	240
45 Met Ile Val Leu Gly Leu Ser Ala Cys Asp His Thr Gln Ala Leu Tyr			
245	250	255	
50 5 Leu Leu His Gly Arg Thr Ile Tyr Gly Asn Lys Lys Met Pro Ser Ser			
260	265	270	
55 Phe Pro Leu Ile Thr Pro Pro Val Leu Ser Leu Phe Phe Ser Ser Arg			
275	280	285	
60 Pro Tyr Ser Ser Gln Pro Lys Arg Asp Leu Glu Leu Ala Val Lys Leu			
290	295	300	
65 Leu Glu Lys Lys Ser Arg Ser Phe Phe Val Ala Ser Ala Gly Phe Pro			
305	310	315	320
70 Ser Glu Val Arg Glu Arg Leu Val Gly Leu Tyr Ala Phe Cys Arg Val			
325	330	335	
75 Thr Asp Asp Leu Ile Asp Ser Pro Glu Val Ser Ser Asn Pro His Ala			
340	345	350	
80 Thr Ile Asp Met Val Ser Asp Phe Leu Thr Leu Leu Phe Gly Pro Pro			
355	360	365	
85 Leu His Pro Ser Gln Pro Asp Lys Ile Leu Ser Ser Pro Leu Leu Pro			
370	375	380	
90 Pro Ser His Pro Ser Arg Pro Thr Gly Met Tyr Pro Leu Pro Pro Pro			
385	390	395	400
95 Pro Ser Leu Ser Pro Ala Glu Leu Val Gln Phe Leu Thr Glu Arg Val			
405	410	415	
100 Pro Val Gln Tyr His Phe Ala Phe Arg Leu Leu Ala Lys Leu Gln Gly			
420	425	430	
105 Leu Ile Pro Arg Tyr Pro Leu Asp Glu Leu Leu Arg Gly Tyr Thr Thr			
435	440	445	

Asp Leu Ile Phe Pro Leu Ser Thr Glu Ala Val Gln Ala Arg Lys Thr
 450 455 460
 Pro Ile Glu Thr Thr Ala Asp Leu Leu Asp Tyr Gly Leu Cys Val Ala
 , 465 470 475 480
 Gly Ser Val Ala Glu Leu Leu Val Tyr Val Ser Trp Ala Ser Ala Pro
 485 490 495
 10 Ser Gln Val Pro Ala Thr Ile Glu Glu Arg Glu Ala Val Leu Val Ala
 500 505 510
 Ser Arg Glu Met Gly Thr Ala Leu Gln Leu Val Asn Ile Ala Arg Asp
 515 520 525
 15 Ile Lys Gly Asp Ala Thr Glu Gly Arg Phe Tyr Leu Pro Leu Ser Phe
 530 535 540
 Phe Gly Leu Arg Asp Glu Ser Lys Leu Ala Ile Pro Thr Asp Trp Thr
 20 545 550 555 560
 Glu Pro Arg Pro Gln Asp Phe Asp Lys Leu Leu Ser Leu Ser Pro Ser
 565 570 575
 25 Ser Thr Leu Pro Ser Ser Asn Ala Ser Glu Ser Phe Arg Phe Glu Trp
 580 585 590
 Lys Thr Tyr Ser Leu Pro Leu Val Ala Tyr Ala Glu Asp Leu Ala Lys
 595 600 605
 30 His Ser Tyr Lys Gly Ile Asp Arg Leu Pro Thr Glu Val Gln Ala Gly
 610 615 620
 Met Arg Ala Ala Cys Ala Ser Tyr Leu Leu Ile Gly Arg Glu Ile Lys
 35 625 630 635 640
 Val Val Trp Lys Gly Asp Val Gly Glu Arg Arg Thr Val Ala Gly Trp
 645 650 655
 40 Arg Arg Val Arg Lys Val Leu Ser Val Val Met Ser Gly Trp Glu Gly
 660 665 670
 Gln
 45

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
 50 (A) LENGTH: 1165 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: cDNA
 (iii) HYPOTHETICAL: NO

60 (iv) ANTI-SENSE: NO
 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: *Phaffia rhodozyma*

65 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 141..896
 (D) OTHER INFORMATION: /product= "PRidi"

70 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

5	CTCTCTTTC CTCGACCTCT TGGCAGGCC GTTGAAGACT CGTTTACTCA TACCCCACAT	60
	CTGGCATATA TCACTTCTT CCTTCCAGAA CAAGTTCTGA GTCAACCGAA AAGAAAAGAAG	120
5	GCAGAAGAAA TATATTCTAG ATG TCC ATG CCC AAC ATT GTT CCC CCC GCC Met Ser Met Pro Asn Ile Val Pro Pro Ala	170
	1 5 10	
10	GAG GTC CGA ACC GAA GGA CTC AGT TTA GAA GAG TAC GAT GAG GAG CAG Glu Val Arg Thr Glu Gly Leu Ser Leu Glu Tyr Asp Glu Glu Gln	218
	15 20 25	
15	GTC AGG CTG ATG GAG GAG CGA TGT ATT CTT GTT AAC CGG GAC GAT GTG Val Arg Leu Met Glu Glu Arg Cys Ile Leu Val Asn Pro Asp Asp Val	266
	30 35 40	
20	GCC TAT GGA GAG GCT TCG AAA AAG ACC TGC CAC TTG ATG TCC AAC ATC Ala Tyr Gly Glu Ala Ser Lys Lys Thr Cys His Leu Met Ser Asn Ile	314
	45 50 55	
25	AAC GCG CCC AAG GAC CTC CTC CAC CGA GCA TTC TCC GIG TTT CTC TTC Asn Ala Pro Lys Asp Leu Leu His Arg Ala Phe Ser Val Phe Leu Phe	362
	60 65 70	
30	CGC CCA TCG GAC GGA GCA CTC CTG CTT CAG CGA AGA GCG GAC GAG AAG Arg Pro Ser Asp Gly Ala Leu Leu Gln Arg Arg Ala Asp Glu Lys	410
	75 80 85 90	
35	ATT ACG TTC CCT GGA ATG TGG ACC AAC ACG TGT TGC AGT CAT CCT TTG Ile Thr Phe Pro Gly Met Trp Thr Asn Thr Cys Cys Ser His Pro Leu	458
	95 100 105	
40	AGC ATC AAG GGC GAG GTT GAA GAG GAG AAC CAG ATC GGT GTT CGA CGA Ser Ile Lys Gly Glu Val Glu Glu Asn Gln Ile Gly Val Arg Arg	506
	110 115 120	
45	GCT GCG TCC CGA AAG TTG GAG CAC GAG CTT GGC GTG CCT ACA TCG TCG Ala Ala Ser Arg Lys Leu Glu His Glu Leu Gly Val Pro Thr Ser Ser	554
	125 130 135	
50	ACT CCG CCC GAC TCG TTC ACC TAC CTC ACT AGG ATA CAT TAC CTC GCT Thr Pro Pro Asp Ser Phe Thr Tyr Leu Thr Arg Ile His Tyr Leu Ala	602
	140 145 150	
55	CCG AGT GAC GGA CTC TGG GGA GAA CAC GAG ATC GAC TAC ATT CTC TTC Pro Ser Asp Gly Leu Trp Gly Glu Ile Asp Tyr Ile Leu Phe	650
	155 160 165 170	
60	TCA ACC ACA CCT ACA GAA CAC ACT GGA AAC CCT AAC GAA GTC TCT GAC Ser Thr Thr Pro Thr Glu His Thr Gly Asn Pro Asn Glu Val Ser Asp	698
	175 180 185	
65	ACT CGA TAT GTC ACC AAG CCC GAG CTC CAG GCG ATG TTT GAG GAC GAG Thr Arg Tyr Val Thr Lys Pro Glu Leu Gln Ala Met Phe Glu Asp Glu	746
	190 195 200	
70	TCT AAC TCA TTT ACC CCT TGG TTC AAA TTG ATT GCC CGA GAC TTC CTG Ser Asn Ser Phe Thr Pro Trp Phe Lys Leu Ile Ala Arg Asp Phe Leu	794
	205 210 215	
75	TTT GGC TGG TGG GAT CAA CTT CTC GCC AGA CGA AAT GAA AAG GGT GAG Phe Gly Trp Trp Asp Gln Leu Leu Ala Arg Arg Asn Glu Lys Gly Glu	842
	220 225 230	
80	GTC GAT GCC AAA TCG TTG GAG GAT CTC TCG GAC AAC AAA GTC TGG AAG Val Asp Ala Lys Ser Leu Glu Asp Leu Ser Asp Asn Lys Val Trp Lys	890
	235 240 245 250	
85	ATG TAGTCGACCC TTCTTTCTGT ACAGTCATCT CAGTTGGCT GTTGGTGCT	943
	Met	

TGCTTCTTGC TCTCTTCT ATATATCCTT TTCTCTGCCT GGGTAGACTT GACCTTCTA	1003
CATAGCTTAC GCATACATAC ATAAACTCTA TTCTCTGTC TTTATCTCTC TTCTAAGGGA	1063
5 ATCTTCAAGA TCAATTCTT TTGGGCTAC AACATTCAAG ATCAATATTG CTTTCAGAC	1123
TACAAAAAAA AAAAAAAA ACTUGAGGGG GGGCCGGTA CC	1165

10 (2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 251 amino acids
 (B) TYPE: amino acid
 15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

20 Met Ser Met Pro Asn Ile Val Pro Pro Ala Glu Val Arg Thr Glu Gly	1	5	10	15
25 Leu Ser Leu Glu Glu Tyr Asp Glu Glu Gln Val Arg Leu Met Glu Glu	20	25	30	
30 Arg Cys Ile Leu Val Asn Pro Asp Asp Val Ala Tyr Gly Glu Ala Ser	35	40	45	
35 Lys Lys Thr Cys His Leu Met Ser Asn Ile Asn Ala Pro Lys Asp Leu	50	55	60	
40 Leu His Arg Ala Phe Ser Val Phe Leu Phe Arg Pro Ser Asp Gly Ala	65	70	75	80
45 35 Leu Leu Leu Gln Arg Arg Ala Asp Glu Lys Ile Thr Phe Pro Gly Met	85	90	95	
50 Trp Thr Asn Thr Cys Cys Ser His Pro Leu Ser Ile Lys Gly Glu Val	100	105	110	
55 Glu Glu Glu Asn Gln Ile Gly Val Arg Arg Ala Ala Ser Arg Lys Leu	115	120	125	
60 45 Glu His Glu Leu Gly Val Pro Thr Ser Ser Thr Pro Pro Asp Ser Phe	130	135	140	
65 Thr Tyr Leu Thr Arg Ile His Tyr Leu Ala Pro Ser Asp Gly Leu Trp	145	150	155	160
70 55 Gly Glu His Glu Ile Asp Tyr Ile Leu Phe Ser Thr Thr Pro Thr Glu	165	170	175	
75 His Thr Gly Asn Pro Asn Glu Val Ser Asp Thr Arg Tyr Val Thr Lys	180	185	190	
80 Pro Glu Leu Gln Ala Met Phe Glu Asp Glu Ser Asn Ser Phe Thr Pro	195	200	205	
85 60 Trp Phe Lys Leu Ile Ala Arg Asp Phe Leu Phe Gly Trp Trp Asp Gln	210	215	220	
90 Leu Leu Ala Arg Arg Asn Glu Lys Gly Glu Val Asp Ala Lys Ser Leu	225	230	235	240
95 65 Glu Asp Leu Ser Asp Asn Lys Val Trp Lys Met	245	250		

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3550 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Phaffia rhodozyma*
- (B) STRAIN: CBS 6938

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 941..966

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 967..1077

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 1078..1284

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 1285..1364

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 1365..1877

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 1878..1959

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 1960..2202

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 2203..2292

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 2293..3325

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: join(941..966, 1078..1284, 1365..1877, 1960..2202, 2293..3325)

(D) OTHER INFORMATION: /product= "PRGcrtB GB"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GGAATTCCAG TTTGCCCTT GACGAGAAAG GACACTGGGT TGGAAAGAGA AGATGGTACG 60

TCTCTCTCCA CCTTGAATGT GTTGTCTACT AGACATGTTT GACACCTAA TGGATTTCTT 120

TCCACTTTGA CTTTGAACT ATGGTGTTTG GGGATCCCC AAAATCATTA GCTTCTACTTT 180

CAGCTCATTA CCTGATCTC ATCTTACTAC CAGGTGTTGC ATTCTCACCT ACGGCCTCTT 240

1	CITTTGTCCTC TCGACTGGGC CATGGAAAAG GATATTACGA TAAATACATC ACTCAGIATC	300
2	GGTCGATCTG TGCAGGCAAG AATCGACCG TCCGAAGCTG AGTACGGCTC TTCTCTTTTC	360
3	TOGATAACCCA ACGGAOGCTA TTTTGTGACA GAAGGATGAG ACTATCCAAC AGCTCAAACA	420
4	AACTAACGCT CTGATTAAT CACCGCTCA ACTTATTGCT CAACTCAGTT GGACTGGCGC	480
5	TGAAAGAACA GTTCTTAGAC AAAAACATGG TCCCTATAGG AGAAATGGAT GCGAATCTGG	540
10	ATGAAGTGTG CGTGGAGAT CACCTGAGGA CATTATCCGA GGACAAATTAA CTACTTAAGA	600
11	TATATACATG ATTTATGTOG ATCGGCATCC AGCCGGGGAT TGATGGCTG ATGGCCGGAA	660
12	ATGIGATGAT GGTGAAACT CGACCTCTCT TTTTGTGTC ATCTCTCAT CCTCTCTCTC	720
13	TCTTCTCTACT GACATCCATC TCCAACCTGTC TAGATCAGIT CGGAAACAAG AAGTGGACAC	780
14	AGAGAGATCT TTGCTGAAGA GTTGTATTCC AGAAAGGGAA AACAAAGGAA AGAAGGCGG	840
20	AAGCACATCA CCAACTTCAG CAAGCCGGTC CAGCCCGATC TCGGATAGAC ATCATCTTAC	900
21	CCAACCTGTA TCATCCCCAA CAGATAGAGT TTTTGTGCA ATG ACG GCT CTC GCA	955
22	Met Thr Ala Leu Ala	
23	1 5	
24	TAT TAC CAG AT GTTGTGTCCTC ATACCTCTTC TTGGTTTGTG ACACCACTCA	1006
25	Tyr Tyr Gln Ile	
30	TGIGTGCTATA TGIGIGTGCG TTCTTCCAAA TCTTTCAATG ACTAACATCT TTACCGTGCT	1066
31	CTCTCTCTTA G C CAT CTG ATC TAT ACT CTC CCA ATT CTT GGT CTT CTC	1114
32	His Leu Ile Tyr Thr Leu Pro Ile Leu Gly Leu Leu	
33	10 15 20	
34	GGC CTG CTC ACT TCC CCG ATT TTG ACA AAA TTT GAC ATC TAC AAA ATA	1162
35	Gly Leu Leu Thr Ser Pro Ile Leu Thr Lys Phe Asp Ile Tyr Lys Ile	
36	25 30 35	
40	TCG ATC CTC GTC ATT GCG TTT AGT GCA ACC ACA CCA TGG GAC TCA	1210
41	Ser Ile Leu Val Phe Ile Ala Phe Ser Ala Thr Thr Pro Trp Asp Ser	
42	40 45 50	
45	TGG ATC ATC AGA AAT GGC GCA TGG ACA TAT CCA TCA GCG GAG AGT GGC	1258
46	Trp Ile Ile Arg Asn Gly Ala Trp Thr Tyr Pro Ser Ala Glu Ser Gly	
47	55 60 65	
50	CAA GGC GTG TTT GGA ACG TTT CTA GA GTTAGTCGAC CGTTAACT	1304
51	Gln Gly Val Phe Gly Thr Phe Leu Asp	
52	70 75	
55	CTTAGCOGGCG CGTGTTTCC CGGATTACAT TTAACATCTG AATTTATCCC TGATCAACAG	1364
56	T GTT CCA TAT GAA GAG TAC GCT TTC TTT GTC ATT CAA ACC GTC ATC	1410
57	Val Pro Tyr Glu Glu Tyr Ala Phe Phe Val Ile Gln Thr Val Ile	
58	80 85 90	
60	ACC GGC TTG GTC TAC GTC TTG GCA ACT AGG CAC CTT CTC CCA TCT CTC	1458
61	Thr Gly Leu Val Tyr Val Leu Ala Thr Arg His Leu Leu Pro Ser Leu	
62	95 100 105	
65	GCG CTT CCC AAG ACT AGA TCG TCC GCG CTT TCT CTC GCG CTC AAG GCG	1506
66	Ala Leu Pro Lys Thr Arg Ser Ser Ala Leu Ser Leu Ala Leu Lys Ala	
67	110 115 120 125	
70	CTC ATC CCT CTG CCC ATT ATC TAC CTA TTT ACC GCT CAC CCC AGC CCA	1554
71	Leu Ile Pro Leu Pro Ile Ile Tyr Leu Phe Thr Ala His Pro Ser Pro	
72	130 135 140	

145	TCG CCC GAC CCG CTC GTG ACA GAT CAC TAC TTC TAC ATG CGG GCA CTC Ser Pro Asp Pro Leu Val Thr Asp His Tyr Phe Tyr Met Arg Ala Leu	150	155	1602
160	5 TCC TTA CTC ATC ACC CCA CCT ACC ATG CTC TTG GCA GCA TTA TCA GGC Ser Leu Leu Ile Thr Pro Pro Thr Met Leu Leu Ala Ala Leu Ser Gly	165	170	1650
175	10 GAA TAT GCT TTC GAT TGG AAA AGT GGC CGA GCA AAG TCA ACT ATT GCA Glu Tyr Ala Phe Asp Trp Lys Ser Gly Arg Ala Lys Ser Thr Ile Ala	180	185	1698
190	15 GCA ATC ATG ATC CCG ACG GTG TAT CTG ATT TGG GTA GAT TAT GTT GCT Ala Ile Met Ile Pro Thr Val Tyr Leu Ile Trp Val Asp Tyr Val Ala	195	200	1746
210	20 GTC GGT CAA GAC TCT TGG TCG ATC AAC GAT GAG AAG ATT GTA GGG TGG Val Gly Gln Asp Ser Trp Ser Ile Asn Asp Glu Lys Ile Val Gly Trp	215	220	1794
225	25 AGG CTT GGA GGT GTA CTA CCC ATT GAG GAA GCT ATG TTC TTC TTA CTG Arg Leu Gly Val Leu Pro Ile Glu Glu Ala Met Phe Phe Leu Leu	230	235	1842
240	30 ACG AAT CTA ATG ATT GTT CTG GGT CTG TCT GCC TG GTAAAGTTGAT Thr Asn Leu Met Ile Val Leu Gly Leu Ser Ala Cys	245		1887
255	35 CTCATCCTCT CTTCCCTTGG TGAAAAAAAGC TGTGTTGGCTG ATTGCTGOGA ACTCACCCAT			1947
265	40 CGGAATCTGT AG C GAT CAT ACT CAG GCC CTA TAC CTG CTA CAC GGT CGA Asp His Thr Gln Ala Leu Tyr Leu Leu His Gly Arg	270	275	1996
280	45 ACT ATT TAT GGC AAC AAA AAG ATG CCA TCT TCA TTT CCC CTC ATT ACA Thr Ile Tyr Gly Asn Lys Lys Met Pro Ser Ser Phe Pro Leu Ile Thr	285	290	2044
295	50 CCG CCT GTG CTC TCC CTG TTT TTT AGC AGC CGA CCA TAC TCT TCT CAG Pro Pro Val Leu Ser Leu Phe Phe Ser Ser Arg Pro Tyr Ser Ser Gln	300	305	2092
310	55 CCA AAA CGT GAC TTG GAA CTG GCA GTC AAG TTG TTG GAG AAA AAG AGC Pro Lys Arg Asp Leu Glu Leu Ala Val Lys Leu Leu Glu Lys Lys Ser	315	320	2140
330	60 CGG AGC TTT TTT GTT GCC TCG GCT GGA TTT CCT AGC GAA GTT AGG GAG Arg Ser Phe Phe Val Ala Ser Ala Gly Phe Pro Ser Glu Val Arg Glu	335	340	2188
335	65 AGG CTG GTT GGA CT GTGAGCAAGC ATTCTTGTAGG TTGTTGGT CTTCACCTT Arg Leu Val Gly Leu	340	345	2242
350	70 55 CATGTGCATT CGCTGATCPG TTTCTTGGT GATCGGGAC CTGCATACAG A TAC GCA Tyr Ala	355	360	2299
365	75 TTC TGC CGG GTG ACT GAT GAT CTT ATC GAC TCT CCT GAA GTA TCT TCC Phe Cys Arg Val Thr Asp Asp Leu Ile Asp Ser Pro Glu Val Ser Ser	370	375	2347
375	80 AAC CCG CAT GCC ACA ATT GAC ATG GTC TCC GAT TTT CTT ACC CTA CTA Asn Pro His Ala Thr Ile Asp Met Val Ser Asp Phe Leu Thr Leu Leu	380		2395
385	85 TTT GGG CCC CCG CTA CAC CCT TCG CAA CCT GAC AAG ATC CTT TCT TCG Phe Gly Pro Pro Leu His Pro Ser Gln Pro Asp Lys Ile Leu Ser Ser	390		2443
395	90 70 365 370 375 380			

	CCT TTA CTT CCT CCT TCG CAC CCT TCC CGA CCC ACG GGA ATG TAT CCC Pro Leu Leu Pro Pro Ser His Pro Ser Arg Pro Thr Gly Met Tyr Pro 385 390 395	2491
5	CTC CGG CCT CCT TCG CTC TCG CCT GCC GAG CTC GTT CAA TTC CTT Leu Pro Pro Pro Ser Leu Ser Pro Ala Glu Leu Val Gln Phe Leu 400 405 410	2539
10	ACC GAA AGG GTT CCC GTT CAA TAC CAT TTC GCC TTC AGG TTG CTC GCT Thr Glu Arg Val Pro Val Gln Tyr His Phe Ala Phe Arg Leu Leu Ala 415 420 425	2587
15	AAG TTG CAA GGG CTG ATC CCT CGA TAC CCA CTC GAC GAA CTC CTT AGA Lys Leu Gln Gly Leu Ile Pro Arg Tyr Pro Leu Asp Glu Leu Leu Arg 430 435 440	2635
20	GGA TAC ACC ACT GAT CTT ATC TTT CCC TTA TCG ACA GAG GCA GTC CAG Gly Tyr Thr Thr Asp Leu Ile Phe Pro Leu Ser Thr Glu Ala Val Gln 445 450 455 460	2683
25	GCT CGG AAG ACG CCT ATC GAG ACC ACA GCT GAC TTG CTG GAC TAT GGT Ala Arg Lys Thr Pro Ile Glu Thr Thr Ala Asp Leu Leu Asp Tyr Gly 465 470 475	2731
30	CTA TGT GTA GCA GGC TCA GTC GCC GAG CTA TTG GTC TAT GTC TCT TGG Leu Cys Val Ala Gly Ser Val Ala Glu Leu Leu Val Tyr Val Ser Trp 480 485 490	2779
35	GCA AGT GCA CCA AGT CAG GTC CCT GCC ACC ATA GAA GAA AGA GAA GCT Ala Ser Ala Pro Ser Gln Val Pro Ala Thr Ile Glu Glu Arg Glu Ala 495 500 505	2827
40	GTG TTA GTG GCA AGC CGA GAG ATG GGA ACT GCC CTT CAG TTG GTG AAC Val Leu Val Ala Ser Arg Glu Met Gly Thr Ala Leu Gln Leu Val Asn 510 515 520	2875
45	ATT GCT AGG GAC ATT AAA GGG GAC GCA ACA GAA GGG AGA TTT TAC CTA Ile Ala Arg Asp Ile Lys Gly Asp Ala Thr Glu Gly Arg Phe Tyr Leu 525 530 535 540	2923
50	CCA CTC TCA TTC TTT GGT CTT CGG GAT GAA TCA AAG CTT GCG ATC CCG Pro Leu Ser Phe Gly Leu Arg Asp Glu Ser Lys Leu Ala Ile Pro 545 550 555	2971
55	ACT GAT TGG ACG GAA CCT CGG CCT CAA GAT TTC GAC AAA CTC CTC AGT Thr Asp Trp Thr Glu Pro Arg Pro Gln Asp Phe Asp Lys Leu Leu Ser 560 565 570	3019
60	CTA TCT CCT TCG TCC ACA TTA CCA TCT TCA AAC GCC TCA GAA AGC TTC Leu Ser Pro Ser Ser Thr Leu Pro Ser Ser Asn Ala Ser Glu Ser Phe 575 580 585	3067
65	CGG TTC GAA TGG AAG ACG TAC TCG CTT CCA TTA GTC GCC TAC GCA GAG Arg Phe Glu Trp Lys Thr Tyr Ser Leu Pro Leu Val Ala Tyr Ala Glu 590 595 600	3115
70	GAT CTT GCC AAA CAT TCT TAT AAG GGA ATT GAC CGA CTT CCT ACC GAG Asp Leu Ala Lys His Ser Tyr Lys Gly Ile Asp Arg Leu Pro Thr Glu 605 610 615 620	3163
75	GTG CAA CGG GGA ATG CGA CGG GCT TGC CGG AGC TAC CTA CTG ATC GGC Val Gln Ala Gly Met Arg Ala Ala Cys Ala Ser Tyr Leu Leu Ile Gly 625 630 635	3211
80	CGA GAG ATC AAA GTC GTT TGG AAA GGA GAC GTC GGA GAG AGA AGG ACA Arg Glu Ile Lys Val Val Trp Lys Gly Asp Val Gly Glu Arg Arg Thr 640 645 650	3259
85	GTG GCC GGA TGG AGG AGA GTA CGG AAA GTC TTG AGT GTG GTC ATG AGC	3307

Val Ala Gly Trp Arg Arg Val Arg Lys Val Leu Ser Val Val Met Ser			
655	660	665	
GGA TGG GAA GGG CAG TAAGACAGCG GAAGAAATACC GACAGACAAT GATGAGTGAG	3362		
Gly Trp Glu Gly Gln			
670			
AATAAAAATCA TCTCTAAATCT TCTTTCTCTA GGTGCTCTTT TTGTTTTCT ATTATGACCA	3422		
ACTCTAAAGG AACTGGCTT GCAGATATTT CTCTTCCCCC CAICCTCCCTC CTTCATCG	3482		
TTTGTCTTT CCATTTTGT CGGTTACTA TGTCATTCT TTTCTTGCT TTTCTTATC	3542		
AATCTAGA	3550		

15

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 673 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: protein

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met Thr Ala Leu Ala Tyr Tyr Gln Ile His Leu Ile Tyr Thr Leu Pro				
1	5	10	15	
Ile Leu Gly Leu Leu Gly Leu Leu Thr Ser Pro Ile Leu Thr Lys Phe				
20	25	30		
Asp Ile Tyr Lys Ile Ser Ile Leu Val Phe Ile Ala Phe Ser Ala Thr				
35	40	45		
Thr Pro Trp Asp Ser Trp Ile Ile Arg Asn Gly Ala Trp Thr Tyr Pro				
50	55	60		
Ser Ala Glu Ser Gly Gln Gly Val Phe Gly Thr Phe Leu Asp Val Pro				
65	70	75	80	
Tyr Glu Glu Tyr Ala Phe Phe Val Ile Gln Thr Val Ile Thr Gly Leu				
85	90	95		
Val Tyr Val Leu Ala Thr Arg His Leu Leu Pro Ser Leu Ala Leu Pro				
100	105	110		
Lys Thr Arg Ser Ser Ala Leu Ser Leu Ala Leu Lys Ala Leu Ile Pro				
115	120	125		
Leu Pro Ile Ile Tyr Leu Phe Thr Ala His Pro Ser Pro Ser Pro Asp				
130	135	140		
Pro Leu Val Thr Asp His Tyr Phe Tyr Met Arg Ala Leu Ser Leu Leu				
145	150	155	160	
Ile Thr Pro Pro Thr Met Leu Leu Ala Ala Leu Ser Gly Glu Tyr Ala				
165	170	175		
Phe Asp Trp Lys Ser Gly Arg Ala Lys Ser Thr Ile Ala Ala Ile Met				
180	185	190		
Ile Pro Thr Val Tyr Leu Ile Trp Val Asp Tyr Val Ala Val Gly Gln				
195	200	205		
Asp Ser Trp Ser Ile Asn Asp Glu Lys Ile Val Gly Trp Arg Leu Gly				
210	215	220		
Gly Val Leu Pro Ile Glu Glu Ala Met Phe Phe Leu Leu Thr Asn Leu				

225	230	235	240
Met Ile Val Leu Gly Leu Ser Ala Cys Asp His Thr Gln Ala Leu Tyr			
245	250	255	
5 Leu Leu His Gly Arg Thr Ile Tyr Gly Asn Lys Lys Met Pro Ser Ser			
260	265	270	
10 Phe Pro Leu Ile Thr Pro Pro Val Leu Ser Leu Phe Phe Ser Ser Arg			
275	280	285	
15 Pro Tyr Ser Ser Gln Pro Lys Arg Asp Leu Glu Leu Ala Val Lys Leu			
290	295	300	
20 Leu Glu Lys Lys Ser Arg Ser Phe Phe Val Ala Ser Ala Gly Phe Pro			
305	310	315	320
Ser Glu Val Arg Glu Arg Leu Val Gly Tyr Ala Phe Cys Arg Val Thr			
325	330	335	
25 Asp Asp Leu Ile Asp Ser Pro Glu Val Ser Ser Asn Pro His Ala Thr			
340	345	350	
Ile Asp Met Val Ser Asp Phe Leu Thr Leu Leu Phe Gly Pro Pro Leu			
355	360	365	
30 His Pro Ser Gln Pro Asp Lys Ile Leu Ser Ser Pro Leu Leu Pro Pro			
370	375	380	
35 Ser His Pro Ser Arg Pro Thr Gly Met Tyr Pro Leu Pro Pro Pro			
385	390	395	400
Ser Leu Ser Pro Ala Glu Leu Val Gln Phe Leu Thr Glu Arg Val Pro			
405	410	415	
40 Val Gln Tyr His Phe Ala Phe Arg Leu Leu Ala Lys Leu Gln Gly Leu			
420	425	430	
45 Ile Pro Arg Tyr Pro Leu Asp Glu Leu Leu Arg Gly Tyr Thr Thr Asp			
435	440	445	
Leu Ile Phe Pro Leu Ser Thr Glu Ala Val Gln Ala Arg Lys Thr Pro			
450	455	460	
45 Ile Glu Thr Thr Ala Asp Leu Leu Asp Tyr Gly Leu Cys Val Ala Gly			
465	470	475	480
Ser Val Ala Glu Leu Leu Val Tyr Val Ser Trp Ala Ser Ala Pro Ser			
485	490	495	
50 Gln Val Pro Ala Thr Ile Glu Glu Arg Glu Ala Val Leu Val Ala Ser			
500	505	510	
55 Arg Glu Met Gly Thr Ala Leu Gln Leu Val Asn Ile Ala Arg Asp Ile			
515	520	525	
Lys Gly Asp Ala Thr Glu Gly Arg Phe Tyr Leu Pro Leu Ser Phe Phe			
530	535	540	
60 Gly Leu Arg Asp Glu Ser Lys Leu Ala Ile Pro Thr Asp Trp Thr Glu			
545	550	555	560
65 Pro Arg Pro Gln Asp Phe Asp Lys Leu Leu Ser Leu Ser Pro Ser Ser			
565	570	575	
65 Thr Leu Pro Ser Ser Asn Ala Ser Glu Ser Phe Arg Phe Glu Trp Lys			
580	585	590	
70 Thr Tyr Ser Leu Pro Leu Val Ala Tyr Ala Glu Asp Leu Ala Lys His			
595	600	605	

Ser Tyr Lys Gly Ile Asp Arg Leu Pro Thr Glu Val Gln Ala Gly Met
 610 615 620

Arg Ala Ala Cys Ala Ser Tyr Leu Leu Ile Gly Arg Glu Ile Lys Val
 625 630 635 640

Val Trp Lys Gly Asp Val Gly Glu Arg Arg Thr Val Ala Gly Trp Arg
 645 650 655

10 Arg Val Arg Lys Val Leu Ser Val Val Met Ser Gly Trp Glu Gly Gln
 660 665 670

15

(2) INFORMATION FOR SEQ ID NO:24:

20 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 570 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: cDNA
 (iii) HYPOTHETICAL: NO
 (iv) ANTI-SENSE: NO
 30 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: *Phaffia rhodozyma*
 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 24..500
 (D) OTHER INFORMATION: /product= "PRcDNA10"

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AACACTTGGT TAGTTTGGAC GAC ATG CAG ATC TTC GTC AAG ACC CTC ACG 50
 Met Gln Ile Phe Val Lys Thr Leu Thr
 1 5

45 GGT AAG ACC ATC ACC CTT GAG GTG GAG TCT TCT GAC ACC ATC GAC AAC 98
 Gly Lys Thr Ile Thr Leu Glu Val Ser Ser Asp Thr Ile Asp Asn
 10 15 20 25

50 GTC AAG GCC AAG ATC CAG GAC AAG GAA GGA ATT CCC CCT GAT CAG CAG 146
 Val Lys Ala Lys Ile Gln Asp Lys Glu Gly Ile Pro Pro Asp Gln Gln
 30 35 40

55 CGA CTT ATC TTC GCC GGT AAG CAG CTC GAG GAT GGC CGA ACC CTT TCG 194
 Arg Leu Ile Phe Ala Gly Lys Gln Leu Glu Asp Gly Arg Thr Leu Ser
 45 50 55

60 GAT TAC AAC ATC CAG AAA GAG TCC ACC CTC CAC CTC GTC CTT AGG TTG 242
 Asp Tyr Asn Ile Gln Lys Glu Ser Thr Leu His Leu Val Leu Arg Leu
 65 70

65 CGA GGA GGA GCC AAG AAG CGA AAG AAG CAG TAC ACT ACC CCC AAG 290
 Arg Gly Gly Ala Lys Lys Arg Lys Lys Lys Gln Tyr Thr Thr Pro Lys
 75 80 85

70 AAG ATC AAG CAC AAG CGA AAG AAG GTC AAG ATG GCT ATT CTT AAG TAC 338
 Lys Ile Lys His Lys Arg Lys Lys Val Lys Met Ala Ile Leu Lys Tyr
 90 95 100 105

75 TAC AAG GTC GAC TCT GAT GGA AAG ATC AAG CGA CTT CGT CGA GAG TGC 386

Tyr Lys Val Asp Ser Asp Gly Lys Ile Lys Arg Leu Arg Arg Glu Cys		
110	115	120
CCC CAG CCC CAG TGC GGA GCT GGT ATC TTC ATG GCT TTC CAC TCC AAC		434
5 Pro Gln Pro Gln Cys Gly Ala Gly Ile Phe Met Ala Phe His Ser Asn		
125	130	135
CGA CAG ACT TGC GGA AAG TGT GGT CTT ACC TAC ACC TTC GCC GAG GGA		482
10 Arg Gln Thr Cys Gly Lys Cys Gly Leu Thr Tyr Thr Phe Ala Glu Gly		
140	145	150
ACC CAG CCC TCT GCT TAGATCATCA ATCGTTGTT CCCGAGCGAT CTTTGAGTCT		537
15 Thr Gln Pro Ser Ala		
155		
16 TTTTACATT CTCAAAAAAA AAAAAAAA AAA		570

(2) INFORMATION FOR SEQ ID NO:25:

20 (i) SEQUENCE CHARACTERISTICS:		
(A) LENGTH: 158 amino acids		
(B) TYPE: amino acid		
(D) TOPOLOGY: linear		
25 (ii) MOLECULE TYPE: protein		
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:		
30 Met Gln Ile Phe Val Lys Thr Leu Thr Gly Lys Thr Ile Thr Leu Glu		
1 5 10 15		
Val Glu Ser Ser Asp Thr Ile Asp Asn Val Lys Ala Lys Ile Gln Asp		
20 25 30		
35 Lys Glu Gly Ile Pro Pro Asp Gln Gln Arg Leu Ile Phe Ala Gly Lys		
35 40 45		
40 Gln Leu Glu Asp Gly Arg Thr Leu Ser Asp Tyr Asn Ile Gln Lys Glu		
50 55 60		
55 Ser Thr Leu His Leu Val Leu Arg Leu Arg Gly Gly Ala Lys Lys Arg		
65 70 75 80		
65 85 Lys Lys Lys Gln Tyr Thr Pro Lys Lys Ile Lys His Lys Arg Lys		
85 90 95		
90 Lys Val Lys Met Ala Ile Leu Lys Tyr Tyr Lys Val Asp Ser Asp Gly		
100 105 110		
105 115 Lys Ile Lys Arg Leu Arg Arg Glu Cys Pro Gln Pro Gln Cys Gly Ala		
115 120 125		
120 Gly Ile Phe Met Ala Phe His Ser Asn Arg Gln Thr Cys Gly Lys Cys		
130 135 140		
135 145 Gly Leu Thr Tyr Thr Phe Ala Glu Gly Thr Gln Pro Ser Ala		
145 150 155		

(2) INFORMATION FOR SEQ ID NO:26:

60 (i) SEQUENCE CHARACTERISTICS:		
(A) LENGTH: 303 base pairs		
(B) TYPE: nucleic acid		
(C) STRANDEDNESS: double		
(D) TOPOLOGY: linear		
65 (ii) MOLECULE TYPE: cDNA		

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

5 (vi) ORIGINAL SOURCE:

(A) ORGANISM: *Phaffia rhodozyma*

(ix) FEATURE:

(A) NAME/KEY: CDS

10 (B) LOCATION: 57..278

(D) OTHER INFORMATION: /product= "PRcDNA11"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

15	TTTACACACA AACCTTACCT ACCTTTCAA CAACAAATCA CACCTAAGCT TACATC	56
	ATG GAG TCC ATC AAG ACC TCG ATT TCC AAC GCC GCC AAC TAC GCT TCT	104
	Met Glu Ser Ile Lys Thr Ser Ile Ser Asn Ala Ala Asn Tyr Ala Ser	
20	1 5 10 15	
	GAG ACT GTC AAC CAG GCC ACT AGC GCC ACC TCC AAG GAG GCC AAC AAG	152
	Glu Thr Val Asn Gln Ala Thr Ser Ala Thr Ser Lys Glu Ala Asn Lys	
	20 25 30	
25	GAG GTT GCC AAG GAC TCC AAT GCC GGA GTT GGA ACC CGA ATC AAC GCC	200
	Glu Val Ala Lys Asp Ser Asn Ala Gly Val Gly Thr Arg Ile Asn Ala	
	35 40 45	
30	GGA ATT GAT GCT CTT GGA GAC AAG GCC GAC GAG ACT TCG TCT GAT GCC	248
	Gly Ile Asp Ala Leu Gly Asp Lys Ala Asp Glu Thr Ser Ser Asp Ala	
	50 55 60	
35	AAG TCC AAG GCC TAC AAG CAG AAC ATC TAAGTTATT AGATAGTCGT	295
	Lys Ser Lys Ala Tyr Lys Gln Asn Ile	
	65 70	
40	CCATATTT	303

(2) INFORMATION FOR SEQ ID NO:27:

45 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 73 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

55	Met Glu Ser Ile Lys Thr Ser Ile Ser Asn Ala Ala Asn Tyr Ala Ser	
	1 5 10 15	
	Glu Thr Val Asn Gln Ala Thr Ser Ala Thr Ser Lys Glu Ala Asn Lys	
	20 25 30	
60	Glu Val Ala Lys Asp Ser Asn Ala Gly Val Gly Thr Arg Ile Asn Ala	
	35 40 45	
	Gly Ile Asp Ala Leu Gly Asp Lys Ala Asp Glu Thr Ser Ser Asp Ala	
	50 55 60	
65	Lys Ser Lys Ala Tyr Lys Gln Asn Ile	
	65 70	

70 (2) INFORMATION FOR SEQ ID NO:28:

5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 307 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: cDNA
 (iii) HYPOTHETICAL: NO
 (iv) ANTI-SENSE: NO

15 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: *Phaffia rhodozyma*

20 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 3..227
 (D) OTHER INFORMATION: /product= "PRcDNA18"

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

25	AC CCT TCC ATC GAG TCT GAG GCC CGA CAA CAC AAG CTC AAG AGG CTT	47
	Pro Ser Ile Glu Ser Glu Ala Arg Gln His Lys Leu Lys Arg Leu	
	1 5 10 15	
30	GTG CAG AGC CCC AAC TCT TTC TTC ATG GAC GTC AAG TGC CCT GGT TGC	95
	Val Gln Ser Pro Asn Ser Phe Phe Met Asp Val Lys Cys Pro Gly Cys	
	20 25 30	
35	TTC CAG ATC ACC ACC GTG TTC TCG CAC GCT TCC ACT GCC GTT CAG TGT	143
	Phe Gln Ile Thr Thr Val Phe Ser His Ala Ser Thr Ala Val Gln Cys	
	35 40 45	
40	GGA TCG TGC CAG ACC ATC CTC TGC CAG CCC CGG GGA GGA AAG GCT CGA	191
	Gly Ser Cys Gln Thr Ile Leu Cys Gln Pro Arg Gly Gly Lys Ala Arg	
	50 55 60	
45	CTT ACC GAG GGA TGC TCT TTC CGA CGA AAG AAC TAAGTTCTG TTATCGGAATG	244
	Leu Thr Glu Gly Cys Ser Phe Arg Arg Lys Asn	
	65 70 75	
50	ATGCATTCAA ATAAAAAGTCA AAAAAAAAAA AAAAAAAAAC TOGAGGGGGG GCCCCGGTACCC	304
	CAA	307

55 (2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 74 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

60 (ii) MOLECULE TYPE: protein

65 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

65	Pro Ser Ile Glu Ser Glu Ala Arg Gln His Lys Leu Lys Arg Leu Val	
	1 5 10 15	
70	Gln Ser Pro Asn Ser Phe Phe Met Asp Val Lys Cys Pro Gly Cys Phe	
	20 25 30	
75	Gln Ile Thr Thr Val Phe Ser His Ala Ser Thr Ala Val Gln Cys Gly	
	35 40 45	

Ser Cys Gln Thr Ile Leu Cys Gln Pro Arg Gly Gly Lys Ala Arg Leu
 50 55 60

Thr Glu Gly Cys Ser Phe Arg Arg Lys Asn
 65 70

(2) INFORMATION FOR SEQ ID NO:30:

10 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 502 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

20 (iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: *Phaffia rhodozyma*

25 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 30..359
 (D) OTHER INFORMATION: /product= "PRcDNA35"

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GTCAGCTCG GCTAAATCG ATTGTAACA ATG TCT GAA CTC GCC CCC TCC TAC 35	Met Ser Glu Leu Ala Ala Ser Tyr 1 5	53
GCC GCT CTT ATC CTC GOC GAC GAG GGT ATT GAG ATC ACC TCT GAG AAG 40	Ala Ala Leu Ile Leu Ala Asp Glu Gly Ile Glu Ile Thr Ser Glu Lys 10 15 20	101
CTC GTC ACT CTC ACC ACC GCC GCC AAG GTT GAG CTT GAG CCC ATC TGG 45	Leu Val Thr Leu Thr Ala Ala Lys Val Glu Leu Glu Pro Ile Trp 25 30 35 40	149
GCC ACT CTC CTT GCC AAG GCC CTC GAG GGA AAG AAC GTC AAG GAG TTG 50	Ala Thr Leu Leu Lys Ala Leu Glu Gly Lys Asn Val Lys Glu Leu 45 50 55	197
CTT TCC AAC GTC GGA TCC GGA GCC GGA GGA GCT GCC CCC GCC GCC GGC 55	Leu Ser Asn Val Gly Ser Gly Ala Gly Gly Ala Ala Pro Ala Ala Ala 60 65 70	245
GTC GCC GGT GGA GCT TCC GCT GAC GOC TCT GAC CCC GCT GAG GAG AAG 60	Val Ala Gly Gly Ala Ser Ala Asp Ala Ser Ala Pro Ala Glu Lys 75 80 85	293
AAG GAG GAG AAG GCT GAG GAC AAG GAG GAG TCT GAC GAC GAC ATG GGT 65	Lys Glu Glu Lys Ala Glu Asp Lys Glu Ser Asp Asp Asp Met Gly 90 95 100	341
TTC GGA CTT TTC GAT TAAACTCCCT CGGCTAAAAA CCTTTTCTT CAACCCCTTC 70	Phe Gly Leu Phe Asp 105 110	396
TCGTGGCATC GTTCACTCGA CGGCTGCGTT TGGTGTCTT TCTTCAGAA TTTTGTCTT 75	GTCTGGTTTC CCAATGGAT NCCTTGAAA TGANGTTCC CAATTG	456
70 (2) INFORMATION FOR SEQ ID NO:31:		502

5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 109 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

10 Met Ser Glu Leu Ala Ala Ser Tyr Ala Ala Leu Ile Leu Ala Asp Glu
 1 5 10 15

Gly Ile Glu Ile Thr Ser Glu Lys Leu Val Thr Leu Thr Thr Ala Ala
 20 25 30

15 Lys Val Glu Leu Glu Pro Ile Trp Ala Thr Leu Leu Ala Lys Ala Leu
 35 40 45

Glu Gly Lys Asn Val Lys Glu Leu Leu Ser Asn Val Gly Ser Gly Ala
 20 50 55 60

Gly Gly Ala Ala Pro Ala Ala Ala Val Ala Gly Gly Ala Ser Ala Asp
 65 70 75 80

25 Ala Ser Ala Pro Ala Glu Glu Lys Glu Glu Lys Ala Glu Asp Lys
 85 90 95

Glu Glu Ser Asp Asp Asp Met Gly Phe Gly Leu Phe Asp
 100 105

30

(2) INFORMATION FOR SEQ ID NO:32:

35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 381 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

45 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: *Phaffia rhodozyma*

(ix) FEATURE:

50 (A) NAME/KEY: CDS
 (B) LOCATION: 7..282
 (D) OTHER INFORMATION: /product= "PRcDNA38"

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TTCAG ATG ACC AAA GGT ACC TCC TCT TTC GGT AAG CGA CAC ACC AAG 48
 Met Thr Lys Gly Thr Ser Ser Phe Gly Lys Arg His Thr Lys
 1 5 10

60 ACC CAC ACC ATC TGC CGA CGA TGT GGT AAC AGG GCT TTC CAC AGG CAG 96
 Thr His Thr Ile Cys Arg Arg Cys Gly Asn Arg Ala Phe His Arg Gln
 15 20 25 30

65 AAG AAG ACC TGT GCC CAG TGT GGA TAC CCT GCC GCC AAG ATG CGA AGC 144
 Lys Lys Thr Cys Ala Gln Cys Gly Tyr Pro Ala Ala Lys Met Arg Ser
 35 40 45

70 TTC AAC TGG GGA GAG AAG GCC AAG AGG AGA AAG ACC ACC GGT ACC GGT 192
 Phe Asn Trp Gly Glu Lys Ala Lys Arg Arg Lys Thr Thr Gly Thr Gly

50	55	60	
CGA ATG CAG CAC CTC AAG GAC GTC TCT CGA CGA TTC AAG AAC GGC TTC			240
Arg Met Gln His Leu Lys Asp Val Ser Arg Arg Phe Lys Asn Gly Phe			
65	70	75	
CGA GAG GGA ACT TCC GCC ACC AAG AAG GTC AAG GCC GAG TAATOGGTTT			289
Arg Glu Gly Thr Ser Ala Thr Lys Lys Val Lys Ala Glu			
80	85	90	
ATCCATCACCC TGGTGATCAG GGCGGGTAAT AATCTTTGT TAGAGACTAT CCACTGTCIG			349
CTGCGCGATC AAACAAAAAA AAAAAAAA AA			381

15 (2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 91 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Met Thr Lys Gly Thr Ser Ser Phe Gly Lys Arg His Thr Lys Thr His
 1 5 10 15

30 Thr Ile Cys Arg Arg Cys Gly Asn Arg Ala Phe His Arg Gln Lys Lys
 20 25 30

35 Thr Cys Ala Gln Cys Gly Tyr Pro Ala Ala Lys Met Arg Ser Phe Asn
 35 40 45

40 Trp Gly Glu Lys Ala Lys Arg Arg Lys Thr Thr Gly Thr Gly Arg Met
 50 55 60

45 Gln His Leu Lys Asp Val Ser Arg Arg Phe Lys Asn Gly Phe Arg Glu
 65 70 75 80

50 Gly Thr Ser Ala Thr Lys Val Lys Ala Glu
 85 90

45 (2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 473 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

55 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Phaffia rhodozyma

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 19..321
 (D) OTHER INFORMATION: /product= "PRCDNA46"

70 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CTCAAGAAGA AACTCGCC ATG CCT ACC CGA TTC TCC AAC ACC CGA AAG CAC Met Pro Thr Arg Phe Ser Asn Thr Arg Lys His 1 5 10	51
5 AGA GGA CAC GTC TCT GCC GGT CAC GGT CGT GTG GGA AAG CAC AGA AAG Arg Gly His Val Ser Ala Gly His Gly Arg Val Gly Lys His Arg Lys 15 20 25	99
10 CAC CCA GGA CGA CGA GGT CTT GCT GGA GGA CAG CAC CAC CGA ACC His Pro Gly Gly Arg Gly Leu Ala Gly Gly Gln His His His Arg Thr 30 35 40	147
15 AAC TTC GAT AAG TAC CAC CCT GGA TAC TTC GGA AAG GTC GGA ATG AGG Asn Phe Asp Lys Tyr His Pro Gly Tyr Phe Gly Lys Val Gly Met Arg 45 50 55	195
20 CAC TTC CAC CTT ACC CGA NAC TCT TCC TGG TGC CCT ACC GTC AAC ATT His Phe His Leu Thr Arg Xaa Ser Ser Trp Cys Pro Thr Val Asn Ile 60 65 70 75	243
25 GAC NAG CTC TGG ACT CTC GTC CCC GCT GAG GAG AAG AAG GAC TTC CCC Asp Xaa Leu Trp Thr Leu Val Pro Ala Glu Glu Lys Lys Asp Phe Pro 80 85 90	291
30 AAC CAG GCT CGA CCT CGT CCC CGT TGT TGACACTTTG GCTCTCGGTT Asn Gln Ala Arg Pro Arg Pro Arg Cys 95 100	338
35 ACGGCAAATGT TCTTGGCAAG GGTCTACTTC CCCAGATCCC TTTAATCGTC AAGGCCGAT TCNTTTCCGC TCTTGGCGAG AANAANATCN ANGANGCTGG TTGGAATTCC TCTCCCCCTT GTTOCCCCCC TAANG	398
40	458
45	473

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
 40 (A) LENGTH: 100 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Met Pro Thr Arg Phe Ser Asn Thr Arg Lys His Arg Gly His Val Ser 1 5 10 15
50 Ala Gly His Gly Arg Val Gly Lys His Arg Lys His Pro Gly Gly Arg 20 25 30
55 Gly Leu Ala Gly Gly Gln His His His Arg Thr Asn Phe Asp Lys Tyr 35 40 45
60 His Pro Gly Tyr Phe Gly Lys Val Gly Met Arg His Phe His Leu Thr 50 55 60
65 Arg Xaa Ser Ser Trp Cys Pro Thr Val Asn Ile Asp Xaa Leu Trp Thr 65 70 75 80
70 Leu Val Pro Ala Glu Glu Lys Lys Asp Phe Pro Asn Gln Ala Arg Pro 85 90 95
75 Arg Pro Arg Cys 100

70 (2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 608 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- 5 (ii) MOLECULE TYPE: cDNA
- 10 (iii) HYPOTHETICAL: NO
- 10 (iv) ANTI-SENSE: NO
- 15 (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: *Phaffia rhodozyma*
- 15 (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 18..453
 - (D) OTHER INFORMATION: /product= "PRcDNA64"
- 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

25	AAGACTCGTC GTTCAGC ATG TCC TCC GTC AAA GCC ACC AAA GGA AAG GGT Met Ser Ser Val Lys Ala Thr Lys Gly Lys Gly 1 5 10	50
30	CCC GCC GCC TCG GCT GAT GTT AAG GCC AAG GCC GCC AAG AAG GCT GCC Pro Ala Ala Ser Ala Asp Val Lys Ala Lys Ala Ala Lys Lys Ala Ala 15 20 25	98
35	CTC AAG GGT ACT CAG TCT ACT TCC ACC AGG AAG GTC CGA ACT TCG GTC Leu Lys Gly Thr Gln Ser Thr Ser Thr Arg Lys Val Arg Thr Ser Val 30 35 40	146
40	TCT TTC CAC CGA CCC AAG ACT CTC CGA CTT CCC CGA GCT CCC AAG TAC Ser Phe His Arg Pro Lys Thr Leu Arg Leu Pro Arg Ala Pro Lys Tyr 45 50 55	194
45	CCC CGA AAG TCG GTC CCT CAC GCC CCT CGA ATG GAT GAG TTC CGA ACT Pro Arg Lys Ser Val Pro His Ala Pro Arg Met Asp Glu Phe Arg Thr 60 65 70 75	242
50	ATC ATC CAC CCC TTG GCT ACC GAG TCC GCC ATG AAG AAG ATT GAG GAG Ile Ile His Pro Leu Ala Thr Glu Ser Ala Met Lys Lys Ile Glu Glu 80 85 90	290
55	CAC AAC ACC CTT GTG TTC ATC GTC GAT GTC AAG TCC AAC AAG CGA CAG His Asn Thr Leu Val Phe Ile Val Asp Val Lys Ser Asn Lys Arg Gln 95 100 105	338
60	ATC AAG GAC GCC GTC AAG AAG CTC TAC GAG GTC GAT ACC GTC CAC NTC Ile Lys Asp Ala Val Lys Leu Tyr Glu Val Asp Thr Val His Xaa 110 115 120	386
65	AAC NCC TTG ATC ACC CCC GCC GGA AGG AAG AAG CTT ACG TCC GAC TTA Asn Xaa Leu Ile Thr Pro Ala Gly Arg Lys Lys Leu Thr Ser Asp Leu 125 130 135	434
70	CCC CCG ACC ACG ACG CTC T TAACTTGCC AACAAAGGCG GCTACATCTA Pro Pro Thr Thr Leu 140 145	483
75	ATGACTCCA TCCCTTGGAT CGGTTCAAGT GTTGGGTTG CATCCGGTTT CAGAGTTGA	543
80	CGACCTTGAA ACTGAAANAC TTGGATGCA TGTGAAAT TCTGAAATA AAAAAAAA AAAAAA	603
85		608

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 145 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Met Ser Ser Val Lys Ala Thr Lys Gly Lys Gly Pro Ala Ala Ser Ala
 1 5 10 15

Asp Val Lys Ala Lys Ala Ala Lys Lys Ala Ala Leu Lys Gly Thr Gln
 15 20 25 30

Ser Thr Ser Thr Arg Lys Val Arg Thr Ser Val Ser Phe His Arg Pro
 20 35 40 45

Lys Thr Leu Arg Leu Pro Arg Ala Pro Lys Tyr Pro Arg Lys Ser Val
 25 50 55 60

Pro His Ala Pro Arg Met Asp Glu Phe Arg Thr Ile Ile His Pro Leu
 30 65 70 75 80

Ala Thr Glu Ser Ala Met Lys Lys Ile Glu Glu His Asn Thr Leu Val
 35 85 90 95

Phe Ile Val Asp Val Lys Ser Asn Lys Arg Gln Ile Lys Asp Ala Val
 40 100 105 110

Lys Lys Leu Tyr Glu Val Asp Thr Val His Xaa Asn Xaa Leu Ile Thr
 45 115 120 125

Pro Ala Gly Arg Lys Lys Leu Thr Ser Asp Leu Pro Pro Thr Thr Thr
 50 130 135 140

Leu
 55 145

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 466 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Phaffia rhodozyma*

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 81..416
- (D) OTHER INFORMATION: /product= "PRcDNA68"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CTTGAACCT CCAACCTCGG CATCAAGCAC TAGTCAGCT CGGCTTAAT CGATTCGIGT 60
 AGCCTTCAA ACTCGTAAAA ATG AAG CAC ATC GCC GCT TAC TTG CTC CTC 110
 70 Met Lys His Ile Ala Ala Tyr Leu Leu

	1	5	10	
5	GCC ACC GGT GGA AAC NCC TCC CCC TCT GCC GCC GAT GTC AAG GCC CTC Ala Thr Gly Gly Asn Xaa Ser Pro Ser Ala Ala Asp Val Lys Ala Leu 15	20	25	158
10	CCT GCC ACC GTC GAC ATC GAG GCT GAT GAC GCC CGA CCT GAG ACC CTC Leu Ala Thr Val Asp Ile Glu Ala Asp Asp Ala Arg Leu Glu Thr Leu 30	35	40	206
15	ATC TCC GAG CCT AAC GGC AAG GAC TTG AAC ACC CTC ATC GCT GAG GGA Ile Ser Glu Leu Asn Gly Lys Asp Leu Asn Thr Leu Ile Ala Glu Gly 45	50	55	254
20	TCC GCC AAG CTC GCT TCC GTC CCC TCC GGA GGA GCC GCC TCT TCC GCT Ser Ala Lys Leu Ala Ser Val Pro Ser Gly Gly Ala Ala Ser Ser Ala 60	65	70	302
25	GCC CCC GCC GCT GGA GGA GCC GCC CCT GCC GCT GAG GAT AAG Ala Pro Ala Ala Ala Gly Gly Ala Ala Pro Ala Ala Glu Asp Lys 75	80	85	350
30	AAG GAG GAG AAG GTC GAG GAC AAG GAG GAG TCT GAC GAC GAC ATG GGT Lys Glu Lys Val Glu Asp Lys Glu Glu Ser Asp Asp Asp Met Gly 95	100	105	398
35	TTC GGA CTT TTC GAT TAAACTCCCT ACACCTTTT CAAACTCTTC GTTGGCTOGA Phe Gly Leu Phe Asp 110			453
40	GGGGGGGGCCC GGT			466

(2) INFORMATION FOR SEQ ID NO:39:

35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 111 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

45	Met Lys His Ile Ala Ala Tyr Leu Leu Leu Ala Thr Gly Gly Asn Xaa 1	5	10	15
50	Ser Pro Ser Ala Ala Asp Val Lys Ala Leu Leu Ala Thr Val Asp Ile 20	25	30	
55	Glu Ala Asp Asp Ala Arg Leu Glu Thr Leu Ile Ser Glu Leu Asn Gly 35	40	45	
60	Lys Asp Leu Asn Thr Leu Ile Ala Glu Gly Ser Ala Lys Leu Ala Ser 50	55	60	
65	Val Pro Ser Gly Gly Ala Ala Ser Ser Ala Ala Pro Ala Ala Ala Gly 65	70	75	80
70	Gly Ala Ala Ala Pro Ala Ala Glu Asp Lys Lys Glu Glu Lys Val Glu 85	90	95	
75	Asp Lys Glu Glu Ser Asp Asp Asp Met Gly Phe Gly Leu Phe Asp 100	105	110	

(2) INFORMATION FOR SEQ ID NO:40:

70 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 570 base pairs

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

10 (iv) ANTI-SENSE: NO

10 (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Phaffia rhodozyma*

15 (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 49..501
- (D) OTHER INFORMATION: /product= "PRcDNA73"

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CTTCTCTCCG TCAAGGCAAA CCTTCAGAAT CCTCTCAAGT CATTCAAC ATG GGA CGA	57
Met Gly Arg	
1	
25 GTC CGC ACC AAA ACC GTC AAG CGA GCT TCG CGA GTG ATG ATC GAG AAG	105
Val Arg Thr Lys Thr Val Lys Arg Ala Ser Arg Val Met Ile Glu Lys	
5 10 15	
30 TTC TAC CCT CGA CTC ACT CTT GAT TTC CAC ACC AAC AAG CGA ATC GCC	153
Phe Tyr Pro Arg Leu Thr Leu Asp Phe His Thr Asn Lys Arg Ile Ala	
20 25 30 35	
35 GAC GAG GTT GCC ATC ATC CCC TCC AAG CGA CTT CGA AAC AAG ATC GCT	201
Asp Glu Val Ala Ile Ile Pro Ser Lys Arg Leu Arg Asn Lys Ile Ala	
40 45 50	
40 GGG TTC ACT ACC CAC TTG ATG AAG CGA ATC CAG AAG GGA CCC GTT CGA	249
Gly Phe Thr Thr His Leu Met Lys Arg Ile Gln Lys Gly Pro Val Arg	
55 60 65	
45 GGT ATC TCC TTC AAG CTT CAG GAG GAG CGA GAG AGG AAG GAT CAG	297
Gly Ile Ser Phe Lys Leu Gln Glu Glu Glu Arg Glu Arg Lys Asp Gln	
70 75 80	
50 TAC GTT CCT GAG GTC TCC GCC CTT GCC GCC CCT GAG CTG GGT TTG GAG	345
Tyr Val Pro Glu Val Ser Ala Leu Ala Ala Pro Glu Leu Gly Leu Glu	
85 90 95	
55 GTT GAC CCC GAC ACC AAG GAT CTT CTC CGA TCC CTT GGC ATG GAC TCC	393
Val Asp Pro Asp Thr Lys Asp Leu Leu Arg Ser Leu Gly Met Asp Ser	
100 105 110 115	
60 ATC AAC GTC CAG GTC TCC GCT CCT ATC TCT TCC TAC GCT GCC CCC GAG	441
Ile Asn Val Gln Val Ser Ala Pro Ile Ser Ser Tyr Ala Ala Pro Glu	
120 125 130	
65 CGA GGT CCC CGA GGT GCC GGA CGA NGT GGA CGA ATC GTC CCC GGA GCT	489
Arg Gly Pro Arg Gly Ala Gly Arg Xaa Gly Arg Ile Val Pro Gly Ala	
135 140 145	
70 GGC CGA TAC TAAAGTGTGTTT CTTCACCCAN GGGATATTTG AINAITCGCT	538
Gly Arg Tyr	
150	
75 AGGCTTGGAAA TTTTTTATC ATTCTTCCIA TA	570

(2) INFORMATION FOR SEQ ID NO:41:

5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 150 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

10 Met Gly Arg Val Arg Thr Lys Thr Val Lys Arg Ala Ser Arg Val Met
 1 5 10 15

Ile Glu Lys Phe Tyr Pro Arg Leu Thr Leu Asp Phe His Thr Asn Lys
 20 25 30

15 Arg Ile Ala Asp Glu Val Ala Ile Ile Pro Ser Lys Arg Leu Arg Asn
 35 40 45

Lys Ile Ala Gly Phe Thr Thr His Leu Met Lys Arg Ile Gln Lys Gly
 20 50 55 60

Pro Val Arg Gly Ile Ser Phe Lys Leu Gln Glu Glu Arg Glu Arg
 65 70 75 80

25 Lys Asp Gln Tyr Val Pro Glu Val Ser Ala Leu Ala Ala Pro Glu Leu
 85 90 95

Gly Leu Glu Val Asp Pro Asp Thr Lys Asp Leu Leu Arg Ser Leu Gly
 100 105 110

30 Met Asp Ser Ile Asn Val Gln Val Ser Ala Pro Ile Ser Ser Tyr Ala
 115 120 125

35 Ala Pro Glu Arg Gly Pro Arg Gly Ala Gly Arg Xaa Gly Arg Ile Val
 130 135 140

40 Pro Gly Ala Gly Arg Tyr
 145 150

40 (2) INFORMATION FOR SEQ ID NO:42:

45 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 373 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

55 (iii) HYPOTHETICAL: NO

60 (iv) ANTI-SENSE: NO

65 (vi) ORIGINAL SOURCE:

(A) ORGANISM: Phaffia rhodozyma

70 (ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 13..324
 (D) OTHER INFORMATION: /product= "PRcDNA76"

65 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

65 CCATCATCCA AC ATG CCT CCC AAA GTC AAG GCC AAG ACC GGT GTC GGT
 Met Pro Pro Lys Val Lys Ala Lys Thr Gly Val Gly
 1 5 10

48

70 AAG ACC CAG AAG AAG AAG TGG TCC AAG GGA AAG GTG AAG GAC AAG

96

(2) INFORMATION FOR SEQ ID NO:43:

30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 103 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

40	Met Pro Pro Lys Val Lys Ala Lys Thr Gly Val Gly Lys Thr Gln Lys	1	5	10	15
	Lys Lys Lys Trp Ser Lys Gly Lys Val Lys Asp Lys Ala Ala His His				
	20	25	30		
45	Val Val Val Asp Gln Ala Thr Tyr Asp Lys Ile Val Lys Glu Val Pro	35	40	45	
	Thr Tyr Lys Leu Ile Ser Gln Ser Ile Leu Ile Asp Arg His Lys Val	50	55	60	
50	Asn Gly Ser Val Ala Arg Ala Ala Ile Arg His Leu Ala Lys Glu Gly	65	70	75	80
	Ser Ile Lys Lys Ile Val His His Asn Gly Gln Trp Ile Tyr Thr Arg	85	90	95	
55	Ala Thr Ala Ala Pro Asp Ala	100			

(2) INFORMATION FOR SEO ID NO:44:

63 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 514 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) ORIGINAL SOURCE:

(A) ORGANISM: *Phaffia rhodozyma*

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 13..435

(D) OTHER INFORMATION: /product= "PRcDNA78"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

15	AAAAAAAGCCA AT ATG CTT ATC TCT AAA CAG AAC AGG AGG GCC ATC TTC Met Leu Ile Ser Lys Gln Asn Arg Arg Ala Ile Phe 1 5 10	48
20	GAG AAC CTC TTC AAG GAG GGA GTT GCC GTC GCC GCC AAG GAC TTC AAC Glu Asn Leu Phe Lys Glu Gly Val Ala Val Ala Ala Lys Asp Phe Asn 15 20 25	96
25	GCT GCC ACC CAC CCC GAG ATT GAG GGT GTC TCC AAC CTT GAG GTC ATC Ala Ala Thr His Pro Glu Ile Glu Gly Val Ser Asn Leu Glu Val Ile 30 35 40	144
30	AAG GCC ATG CAG TCT TTG ACC TCC AAG GGA TAC GTG AAG ACC CAG TTC Lys Ala Met Gln Ser Leu Thr Ser Lys Gly Tyr Val Lys Thr Gln Phe 45 50 55 60	192
35	TCG TGG CAG TAC TAC TAC ACC CTC ACC CCT GAG GGT CTT GAC TAC Ser Trp Gln Tyr Tyr Thr Leu Thr Pro Glu Gly Leu Asp Tyr 65 70 75	240
40	CTC CGA GAG TTC CTC CAC CTT CCC TCC GAG ATT GTC CCC AAC ACT CTC Leu Arg Glu Phe Leu His Leu Pro Ser Glu Ile Val Pro Asn Thr Leu 80 85 90	288
45	AAG CGA CCC ACC CGA CCT GCC AAG GCC CAG GGT CCC GGA GGT GCC TAC Lys Arg Pro Thr Arg Pro Ala Lys Ala Gln Gly Pro Gly Ala Tyr 95 100 105	336
50	CGA GCT CCC CGA GCC GAG GGT GCC GGT CGA GGA GAG TAC CGA CGA CGA Arg Ala Pro Arg Ala Glu Gly Ala Gly Arg Gly Glu Tyr Arg Arg Arg 110 115 120	384
55	GAG GAC GGT GCC GGT GCC TTC GGT GCC GGT CGA GGT CGA CCC CGA GCT Glu Asp Gly Ala Gly Ala Phe Gly Ala Gly Arg Gly Gly Pro Arg Ala 125 130 135 140	432
60	TAAATCCCCAG AGCTTTCTT TTTCGCGTTG CTGGGACTAT GGCAATGAATGA GCTGGCTTGC	492
65	AGAAAAAAAAA AAAAAAAAAA AA	514

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 140 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

Met Leu Ile Ser Lys Gln Asn Arg Arg Ala Ile Phe Glu Asn Leu Phe	
1 5 10 15	

70

Lys Glu Gly Val Ala Val Ala Ala Lys Asp Phe Asn Ala Ala Thr His
 20 25 30

Pro Glu Ile Glu Gly Val Ser Asn Leu Glu Val Ile Lys Ala Met Gln
 35 40 45

Ser Leu Thr Ser Lys Gly Tyr Val Lys Thr Gln Phe Ser Trp Gln Tyr
 50 55 60

Tyr Tyr Tyr Thr Leu Thr Pro Glu Gly Leu Asp Tyr Leu Arg Glu Phe
 65 70 75 80

Leu His Leu Pro Ser Glu Ile Val Pro Asn Thr Leu Lys Arg Pro Thr
 85 90 95

Arg Pro Ala Lys Ala Gln Gly Pro Gly Gly Ala Tyr Arg Ala Pro Arg
 100 105 110

Ala Glu Gly Ala Gly Arg Gly Glu Tyr Arg Arg Arg Glu Asp Gly Ala
 115 120 125

Gly Ala Phe Gly Ala Gly Arg Gly Gly Pro Arg Ala
 130 135 140

25 (2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 437 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

40 (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Phaffia rhodozyma*

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 30..308
- (D) OTHER INFORMATION: /product= "PRcDNA85"

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

50 CTCCTCAAG AAATCAACCA CGGCACATC ATG TCC AAG CGA ACC AAG AAA GTT 53
 Met Ser Lys Arg Thr Lys Lys Val
 1 5

55 GGA ATC ACC GGA AAG TAC GGA GTC CGA TAC GGA GCT TCC CTC CGA AAG 101
 Gly Ile Thr Gly Lys Tyr Gly Val Arg Tyr Gly Ala Ser Leu Arg Lys
 10 15 20

60 ACC GTC AAG AAG NTG GAG GTC TGG CAG CAC GGT ACC TAC ACC TGT GAC 149
 Thr Val Lys Lys Xaa Glu Val Trp Gln His Gly Thr Tyr Thr Cys Asp
 25 30 35 40

65 TTC TGC GGA AAG GAC GCC GTC AAG CGA ACC GCT GTT GGT ATC TGG AAG 197
 Phe Cys Gly Lys Asp Ala Val Lys Arg Thr Ala Val Gly Ile Trp Lys
 45 50 55

70 TGC CGA GGA TGC CGA AAG ACC ACC GCC GGT GGT GCT TGG CAG CTT CAG 245
 Cys Arg Gly Cys Arg Lys Thr Thr Ala Gly Gly Ala Trp Gln Leu Gln
 60 65 70

ACC ACC GGC GCT CTC ACC GTC AAG TCC ACC ACT CGA CGA CTC CGA GAG	293
Thr Thr Ala Ala Leu Thr Val Lys Ser Thr Thr Arg Arg Leu Arg Glu	
75 80 85	
5 CTC AAG GAG GTT TAAATTGAAT TCTGCACAAA GACAAAACIG TTGGGGCGG	345
Leu Lys Glu Val	
90	
10 GAGAGAGTGG ATTCACTCTT TTTTTTGTGATCTGAGG GATGCCATGT CAACCCCTTC	405
10 GTTCCCCAAA AAAAAAAA AAAAAAAA AA	437

(2) INFORMATION FOR SEQ ID NO:47:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 92 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

25 Met Ser Lys Arg Thr Lys Lys Val Gly Ile Thr Gly Lys Tyr Gly Val

1	5	10	15
---	---	----	----

Arg Tyr Gly Ala Ser Leu Arg Lys Thr Val Lys Lys Xaa Glu Val Trp

20	25	30
----	----	----

30 Gln His Gly Thr Tyr Thr Cys Asp Phe Cys Gly Lys Asp Ala Val Lys

35	40	45
----	----	----

35 Arg Thr Ala Val Gly Ile Trp Lys Cys Arg Gly Cys Arg Lys Thr Thr

50	55	60
----	----	----

40 Ala Gly Gly Ala Trp Gln Leu Gln Thr Thr Ala Ala Leu Thr Val Lys

65	70	75	80
----	----	----	----

45 Ser Thr Thr Arg Arg Leu Arg Glu Leu Lys Glu Val

85	90
----	----

(2) INFORMATION FOR SEQ ID NO:48:

45 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 509 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

55 (iii) HYPOTHETICAL: NO

55 (iv) ANTI-SENSE: NO

60 (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Phaffia rhodozyma*

65 (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 35..400
- (D) OTHER INFORMATION: /product= "PrcDNA87"

65 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

70 GGAAGACCTC ACAGCAAGAC TAAGACTCTC AAAC ATG GCT ACC AAG ACC GGC	52
Met Ala Thr Lys Thr Gly	

	1	5	
5	AAG ACT CGA TCC GCT CTC CAG GAC GTC GTT ACT CGG GAG TAC ACC ATC Lys Thr Arg Ser Ala Leu Gln Asp Val Val Thr Arg Glu Tyr Thr Ile 10 15 20		100
10	CAC CTC CAC AAG TAC GTT CAC GGA AGG TCT TTC AAG AAG CGA GCT CCT His Leu His Lys Tyr Val His Gly Arg Ser Phe Lys Lys Arg Ala Pro 25 30 35		148
15	TGG GCT GTC AAG TCC ATC CAG GAG TTT GCT CTC AAG TCG ATG GGA ACC Trp Ala Val Lys Ser Ile Gln Glu Phe Ala Leu Lys Ser Met Gly Thr 40 45 50		196
20	CGA GAT GTC CGA ATT GAC CCC AAG TTG AAC CAG GCC GTC TGG GGA CAG Arg Asp Val Arg Ile Asp Pro Lys Leu Asn Gln Ala Val Trp Gly Gln 55 60 65 70		244
25	GGT GTC AAG AAC CCC CCC AAG CGA CTC CGA ATC CGA CTT GAG CGA AAG Gly Val Lys Asn Pro Pro Lys Arg Leu Arg Ile Arg Leu Glu Arg Lys 75 80 85		292
30	CGA AAC GAC GAG GAG GAT CCT AAG GAC AAG CTC TAC ACT CTT GCT ACC Arg Asn Asp Glu Glu Asp Ala Lys Asp Lys Leu Tyr Thr Leu Ala Thr 90 95 100		340
35	GTC GTC CCC GGA GTC ACC AAC TTC AAG GGT CTC CAA ACC GTT GTC GTT Val Val Pro Gly Val Thr Asn Phe Lys Gly Leu Gln Thr Val Val Val 105 110 115		388
40	GAC ACC GAG TAATTTGTC TTGGATTTTC ATGACGGTCG ATTCAAGCTGT Asp Thr Glu 120		437
45	TTCCTGGGCG CATTCTTCCTT ATGCCACTCTG ATGCCCTTCA CGACCCNTTT TINTTTCTINA		497
50	TAAATAAAAA AA		509

(2) INFORMATION FOR SEQ ID NO:49:

45 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 121 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: protein

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

Met Ala Thr Lys Thr Gly Lys Thr Arg Ser Ala Leu Gln Asp Val Val 1 5 10 15
Thr Arg Glu Tyr Thr Ile His Leu His Lys Tyr Val His Gly Arg Ser 20 25 30
Phe Lys Lys Arg Ala Pro Trp Ala Val Lys Ser Ile Gln Glu Phe Ala 35 40 45
Leu Lys Ser Met Gly Thr Arg Asp Val Arg Ile Asp Pro Lys Leu Asn 50 55 60
Gln Ala Val Trp Gly Gln Gly Val Lys Asn Pro Pro Lys Arg Leu Arg 65 70 75 80
Ile Arg Leu Glu Arg Lys Arg Asn Asp Glu Glu Asp Ala Lys Asp Lys 85 90 95

Leu Tyr Thr Leu Ala Thr Val Val Pro Gly Val Thr Asn Phe Lys Gly
 100 105 110
 Leu Gln Thr Val Val Val Asp Thr Glu
 115 120

(2) INFORMATION FOR SEQ ID NO:50:

10 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 542 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

20 (iv) ANTI-SENSE: NO

(v) ORIGINAL SOURCE:
 (A) ORGANISM: *Phaffia rhodozyma*

25 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 18..443
 (D) OTHER INFORMATION: /product= "PRcDNA95"

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

AGTCGGTATA CATCAAG ATG TCC GTC GTC CAG ACT TTC GGT AAG AAG	50
Met Ser Val Ala Val Gln Thr Phe Gly Lys Lys	
1 5 10	
AAG ACT GCC ACC GCT GTG GCC CAC GCC ACC CCT GGC CGA GGT CTC ATC	98
Lys Thr Ala Thr Ala Val Ala His Ala Thr Pro Gly Arg Gly Leu Ile	
15 20 25	
CGA CTT AAC GGA CAG CCT ATC TCA CTT GOC GAG CCT GCT CTC CTC CGA	146
Arg Leu Asn Gly Gln Pro Ile Ser Leu Ala Glu Pro Ala Leu Leu Arg	
30 35 40	
TAC AAG TAC TAC GAG CCT ATC CTC GTC ATC GGA GCT GAG AAG ATC AAC	194
Tyr Lys Tyr Tyr Glu Pro Ile Leu Val Ile Gly Ala Glu Lys Ile Asn	
45 50 55	
CAG ATC GAC ATC CGA CTC AAG GTC AAG GGT GGA GGA CAC GTC TCC CAG	242
Gln Ile Asp Ile Arg Leu Lys Val Lys Gly Gly Gly His Val Ser Gln	
60 65 70 75	
GTG TAC GCC GTC CGA CAG GCC ATC GGT AAG GCC ATC GTC GCT TAC TAC	290
Val Tyr Ala Val Arg Gln Ala Ile Gly Lys Ala Ile Val Ala Tyr Tyr	
80 85 90	
GCT AAG AAC GTC GAT GCC GCC TCT GCC CTC GAG ATC AAG AAG GCT CTC	338
Ala Lys Asn Val Asp Ala Ala Ser Ala Leu Glu Ile Lys Lys Ala Leu	
95 100 105	
GTC GCC TAC GAC CGA ACC CTC CTC ATC GCC GAT CCC CGA CGA ATG GAG	386
Val Ala Tyr Asp Arg Thr Leu Leu Ile Ala Asp Pro Arg Arg Met Glu	
110 115 120	
CCC AAG AAG TTC GGA GGA CCC GGA GCC CGA CGC GTC CAG AAG TCT	434
Pro Lys Phe Gly Gly Pro Gly Ala Arg Ala Arg Val Gln Lys Ser	
125 130 135	
TAC CGA TAAAAAGTGT TTGCTCTGTG GTCTGGGGGG TCATCTATCC AACATCTTTC	490
Tyr Arg	

140

GAAAAANANIT GTTGGGTCA TATGTCATGC CTCCTTATGG AAAAAAAA AA

542

5 (2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 141 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Met Ser Val Ala Val Gln Thr Phe Gly Lys Lys Lys Thr Ala Thr Ala
1 5 10 1520 Val Ala His Ala Thr Pro Gly Arg Gly Leu Ile Arg Leu Asn Gly Gln
20 25 30Pro Ile Ser Leu Ala Glu Pro Ala Leu Leu Arg Tyr Lys Tyr Tyr Glu
35 40 4525 Pro Ile Leu Val Ile Gly Ala Glu Lys Ile Asn Gln Ile Asp Ile Arg
50 55 6030 Leu Lys Val Lys Gly Gly His Val Ser Gln Val Tyr Ala Val Arg
65 70 75 80Gln Ala Ile Gly Lys Ala Ile Val Ala Tyr Tyr Ala Lys Asn Val Asp
85 90 9535 Ala Ala Ser Ala Leu Glu Ile Lys Lys Ala Leu Val Ala Tyr Asp Arg
100 105 11040 Thr Leu Leu Ile Ala Asp Pro Arg Arg Met Glu Pro Lys Lys Phe Gly
115 120 12545 Gly Pro Gly Ala Arg Ala Arg Val Gln Lys Ser Tyr Arg
130 135 140

45

Claims

1. Recombinant DNA comprising a transcription promoter and a downstream sequence to be expressed, in operable linkage therewith,
 - 5 wherein the transcription promoter comprises a region found upstream of the open reading frame of a highly expressed *Phaffia* gene.
- 10 2. Recombinant DNA according to claim 1, wherein said highly expressed *Phaffia* gene is a glycolytic pathway gene.
- 15 3. Recombinant DNA according to claim 2, wherein said glycolytic pathway gene is a gene coding for Glyceraldehyde-3-Phosphate Dehydrogenase.
4. Recombinant DNA according to claim 1, wherein said highly expressed *Phaffia* gene is a ribosomal protein encoding gene.
5. Recombinant DNA comprising a transcription promoter and a downstream sequence to be expressed, in operable linkage therewith,
 - 20 wherein the transcription promoter comprises a region found upstream of the open reading frame encoding a protein as represented by one of the amino acid sequences depicted in any one of SEQIDNOs: 24 to 50.
- 25 6. A recombinant DNA according to any one of the preceding claims, wherein said downstream sequence to be expressed is heterologous with respect to the transcription promoter sequence.
7. A recombinant DNA according to any one of claims 1 to 6, wherein the downstream sequence comprises an open reading frame coding for a polypeptide responsible for reduced sensitivity against a selective agent.
- 30 8. A recombinant DNA according to claim 7, wherein said selective agent is G418.
9. A recombinant DNA according to any one of claims 1 to 6, wherein the said downstream sequence to be expressed codes for an enzyme involved in the carotenoid biosynthesis pathway.
- 35 10. A recombinant DNA according to claim 9, wherein said downstream sequence to be expressed encodes an enzyme having an activity selected from the group consisting of isopentenyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and lycopene cyclase.

11. A recombinant DNA according to claim 10, wherein said downstream sequence to be expressed encodes an enzyme having an amino acid sequence selected from the one represented by SEQIDNO: 13, SEQIDNO: 15, SEQIDNO: 17, SEQIDNO: 19, SEQIDNO: 21 or SEQIDNO: 23.
12. A recombinant DNA according to any one of the preceding claims, wherein said recombinant DNA comprises further a transcription terminator downstream from the said DNA sequence to be expressed, in operable linkage therewith.
13. A recombinant DNA according to claim 12, wherein the terminator is a GAPDH-encoding gene terminator fragment.
14. A recombinant DNA according to any one of the preceding claims, wherein the recombinant DNA is in the form of a vector capable of replication and/or integration in a host organism.
15. A recombinant DNA according to claim 14, further comprising *Phaffia* ribosomal RNA encoding DNA.
16. A recombinant DNA according to claim 15, which is linearised by cleaving inside the *Phaffia* ribosomal RNA encoding DNA portion.
17. A microorganism harbouring a recombinant DNA according to any one of the preceding claims.
18. A microorganism according to claim 17, which is *Phaffia rhodozyma*.
19. A microorganism according to claim 18, having the recombinant DNA integrated into its genome in an amount of 50 copies or more.
20. An isolated DNA fragment comprising a *Phaffia* GAPDH-gene, or a functional fragment thereof.
21. Use of a functional fragment according to claim 20 for making a recombinant DNA construct.
22. The use according to claim 21, wherein said fragment is a regulatory region normally located upstream or downstream of the open reading frame coding for GAPDH in *Phaffia rhodozyma*.
23. A method for obtaining a transformed *Phaffia* strain, comprising the steps of
 - (a) contacting cells or protoplasts of a *Phaffia* strain with recombinant DNA under conditions conducive to uptake thereof,

said recombinant DNA comprising a transcription promoter and a downstream sequence to be expressed in operable linkage therewith,

(b) identifying *Phaffia rhodozyma* cells or protoplasts having obtained the said recombinant DNA in expressible form,

5 wherein the recombinant DNA is one according to any one of the preceding claims.

24. A method according to claim 23, comprising the additional step of providing an electropulse after contacting of *Phaffia* cells or protoplasts with the said recombinant DNA.

10 25. A transformed *Phaffia* strain obtainable by a method according to any one of the preceding claims, said strain, upon cultivation, being capable of expression of the said downstream sequence, as a consequence of transformation with the said recombinant DNA.

15 26. A transformed *Phaffia* strain according to claim 25, wherein the said downstream sequence codes for a pharmaceutical protein.

27. A transformed *Phaffia* strain according to any one of claims 24 to 26, wherein the said *Phaffia* strain contains at least 10, preferably at least 50, copies of the said recombinant DNA integrated into its genome.

20 28. An isolated DNA sequence coding for an enzyme involved in the carotenoid biosynthetic pathway of *Phaffia rhodozyma*.

25 29. An isolated DNA sequence according to claim 28, wherein said enzyme has an activity selected from isopentenyl pyrophosphate isomerase activity, geranylgeranyl pyrophosphate synthase activity, phytoene synthase activity, phytoene desaturase activity and lycopene cyclase activity.

30 30. An isolated DNA sequence coding for an enzyme having an amino acid sequence selected from the one represented by SEQIDNO: 13, SEQIDNO: 15, SEQIDNO: 17, SEQIDNO: 19, SEQIDNO: 21 or SEQIDNO: 23.

35 31. An isolated DNA sequence coding for a variant of an enzyme according to claim 30, said variant being selected from (i) an allelic variant, (ii) an enzyme having one or more amino acid additions, deletions and/or substitutions and still having the stated enzymatic activity.

32. An isolated DNA sequence encoding an enzyme involved in the carotenoid biosynthesis pathway selected from:

(i) a DNA sequence as represented in SEQIDNO: 12, SEQIDNO: 14, SEQIDNO: 16 SEQIDNO: 18; SEQIDNO: 20, r SEQIDNO: 22.

(ii) an isocoding variant of the DNA sequence represented in SEQIDNO: 12, SEQIDNO: 14, SEQIDNO: 16, SEQIDNO: 18, SEQIDNO: 20 or SEQIDNO: 22;

(iii) an allelic variant of a DNA sequence as represented in SEQIDNO: 12, SEQIDNO: 14, SEQIDNO: 16, SEQIDNO: 18; SEQIDNO: 20 or SEQIDNO: 22;

5 (iv) a DNA sequence capable, when bound to nitrocellulose filter and after incubation under hybridising conditions and subsequent washing, of specifically hybridising to a radio-labelled DNA fragment having the sequence represented in SEQIDNO: 12, SEQIDNO: 14, SEQIDNO: 16, SEQIDNO: 18, SEQIDNO: 20 or SEQIDNO: 22, as detectable by autoradiography of the filter after incubation and washing, wherein said incubation under hybridising conditions and subsequent washing is performed by incubating

10 the filter-bound DNA at a temperature of at least 50°C, preferably at least 55°C, in the presence of a solution of the said radio-labeled DNA in 0.3 M NaCl, 40 mM Tris-HCl, 2 mM EDTA, 0.1% SDS, pH 7.8 for at least one hour, whereafter the filter is washed at least twice for about 20 minutes in 0.3 M NaCl, 40 mM Tris-HCl, 2 mM EDTA, 0.1% SDS, pH 7.8, at a temperature of 50°C, preferably at least 55°C, prior to autoradiography.

15

33. Recombinant DNA comprising an isolated DNA sequence according to any one of claims 27 to 32.

34. Recombinant DNA according to claim 33, wherein said isolated DNA sequence is operably linked to a transcription promoter capable of being expressed in a suitable host, said isolated DNA sequence optionally being linked also to a transcription terminator functional in the said host.

20

35. Recombinant DNA according to claim 34, wherein said host is a *Phaffia* strain.

25 36. Recombinant DNA according to any one of claims 33 to 35, wherein the transcription promoter is from a glycolytic pathway gene present in *Phaffia*.

30

37. Recombinant DNA according to claim 36, wherein said glycolytic pathway gene is a gene coding for Glyceraldehyde-3-Phosphate Dehydrogenase.

38. Recombinant DNA according to any one of claims 33 to 35, wherein the transcription promoter is from a ribosomal protein encoding gene.

35 39. Recombinant DNA according to any one of claims 33 to 35, wherein the transcription promoter comprises a region found upstream of the open reading frame encoding a protein as represented by one of the amino acid sequences depicted in any one of SEQIDNOs: 24 to 50.

40. Recombinant DNA according to any one of claims 27 to 39, wherein said recombinant DNA comprises further a transcription terminator downstream from the said heterologous DNA sequence to be expressed, in operable linkage therewith, which terminator is a *Phaffia* transcription terminator.

3 41. Recombinant DNA according to any one of claims 27 to 40, being in the form of a vector.

42. Use of a vector according to claim 41 to transform a host.

43. Use according to claim 19, wherein the host is a *Phaffia* strain.

10 44. A host obtainable by transformation, optionally of an ancestor, using a recombinant DNA according to any one of claims 27 to 41.

45. A host according to claim 44, which is a *Phaffia* strain, preferably a *Phaffia rhodozyma* strain.

15 46. A transformed *Phaffia rhodozyma* strain which is capable of overexpressing a DNA sequence encoding an enzyme involved in the carotenoid biosynthesis pathway.

20 47. A transformed *Phaffia rhodozyma* strain according to claim 46, which produces increased amounts of astaxanthin relative to its untransformed ancestor.

48. A method for producing an enzyme involved in the carotenoid biosynthesis pathway, by culturing a host according to claim 44 or 45, under conditions conducive to the production of said enzyme.

25 49. A method for producing a carotenoid, characterised in that a host according to any one of claims 44 to 47 is cultivated under conditions conducive to the production of the carotenoid.

50. A method according to claim 49, wherein the carotenoid is astaxanthin.

30 51. A method for producing a pharmaceutical protein by culturing a transformed *Phaffia* strain according to claim 26 under conditions conducive to the production of the said protein.

52. A method for the isolation of a promoter from a highly expressed gene in *Phaffia*, comprising 35 the steps of:

- (a) making a cDNA library on mRNA isolated from a *Phaffia* strain grown under desired conditions;
- (b) determining (part of) the nucleotide sequence of the (partial) cDNAs obtained in step (a);
- (c) comparing the obtained sequence data in step (b) to known sequence data;

- (d) cloning amplifying putative promoter fragments of the gene located either directly upstream of the open reading frame or directly upstream of the transcription start site of the gene corresponding to the expressed cDNA, and
- (e) verifying whether the promoter sequences obtained give high-level expression in a *Phaffia* strain, by
expressing a suitable marker under the control of the promoter in a transformed *Phaffia* strain.

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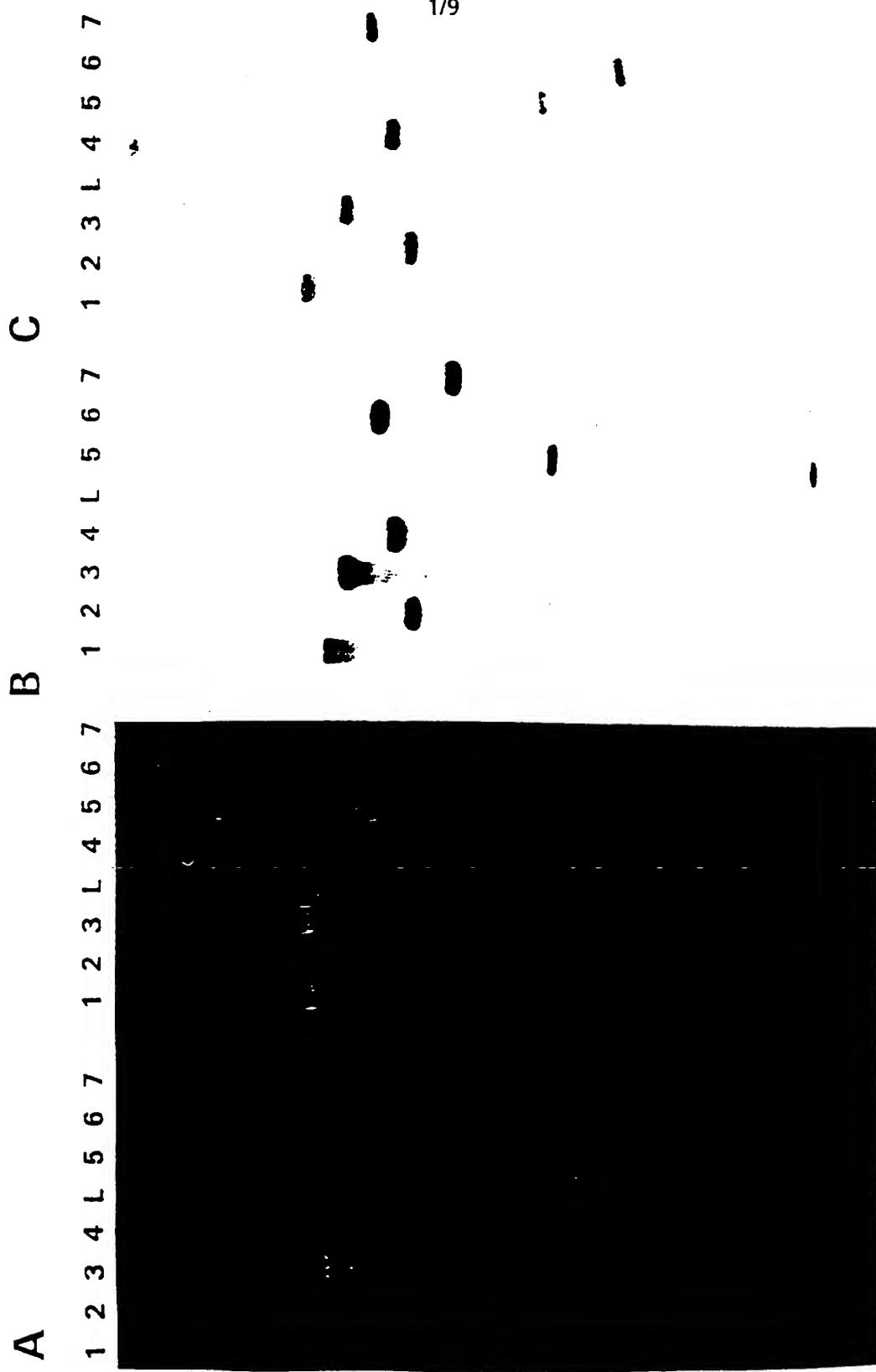


FIG. 1

SUBSTITUTE SHEET (RULE 26)

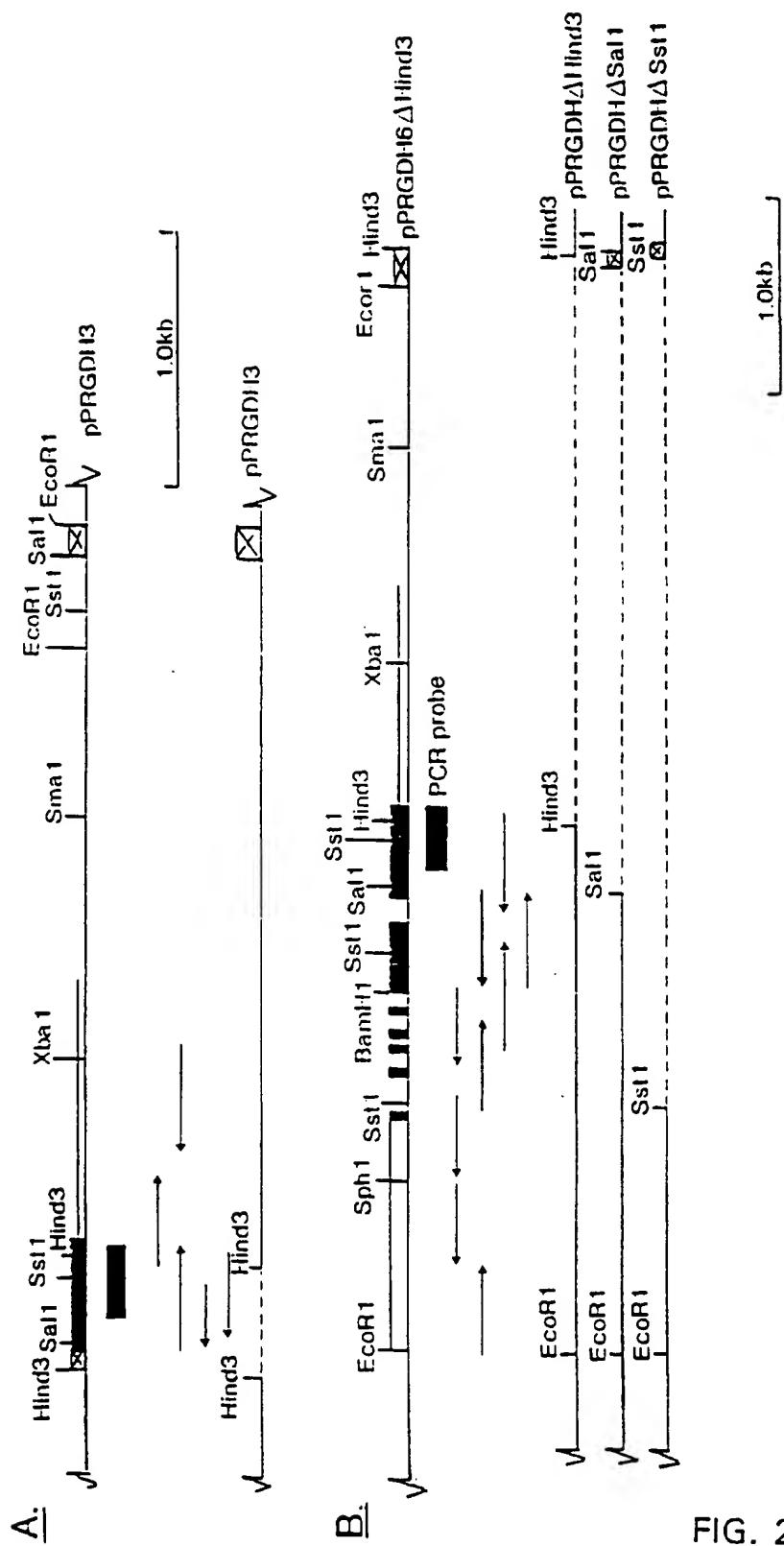


FIG. 2

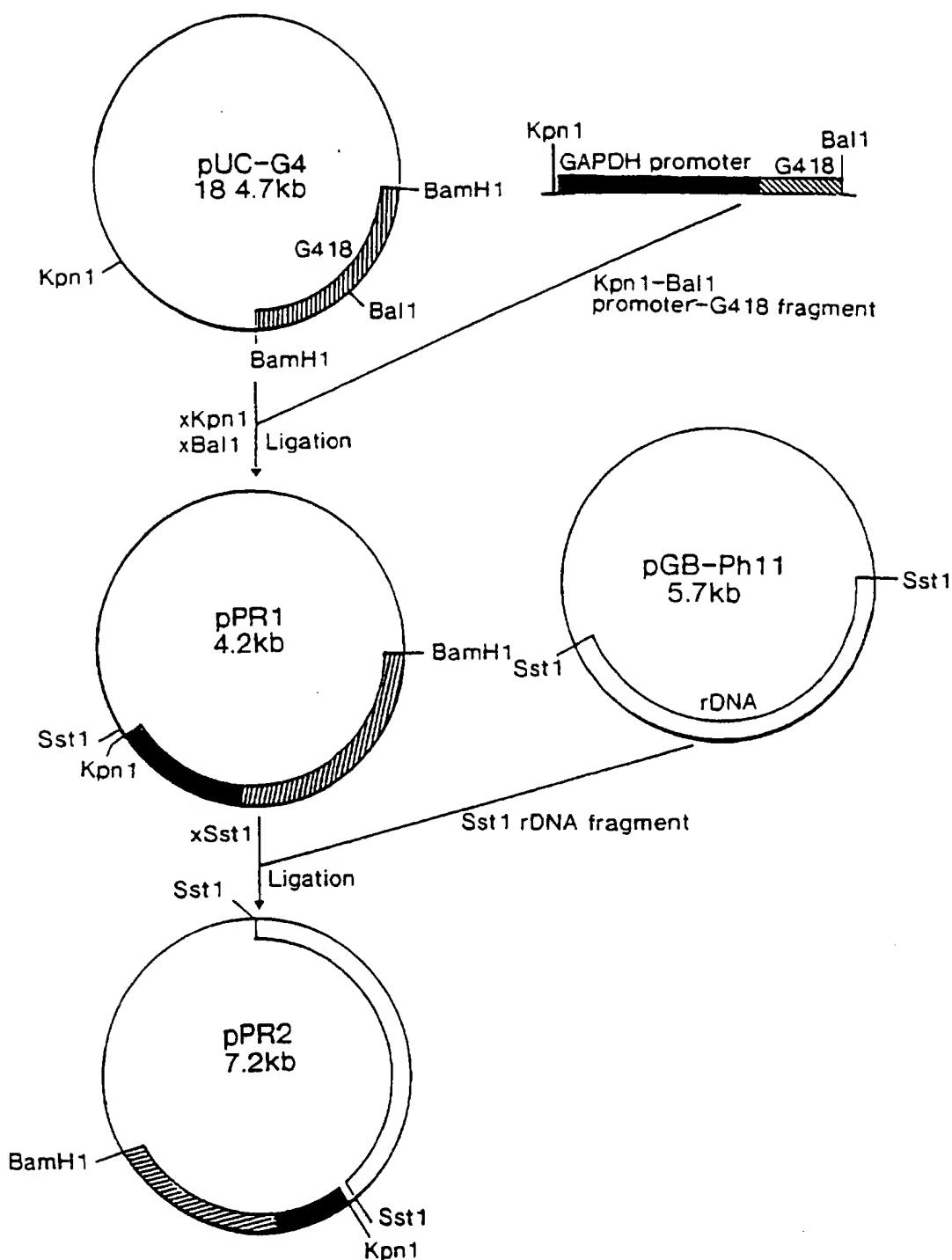


FIG. 3

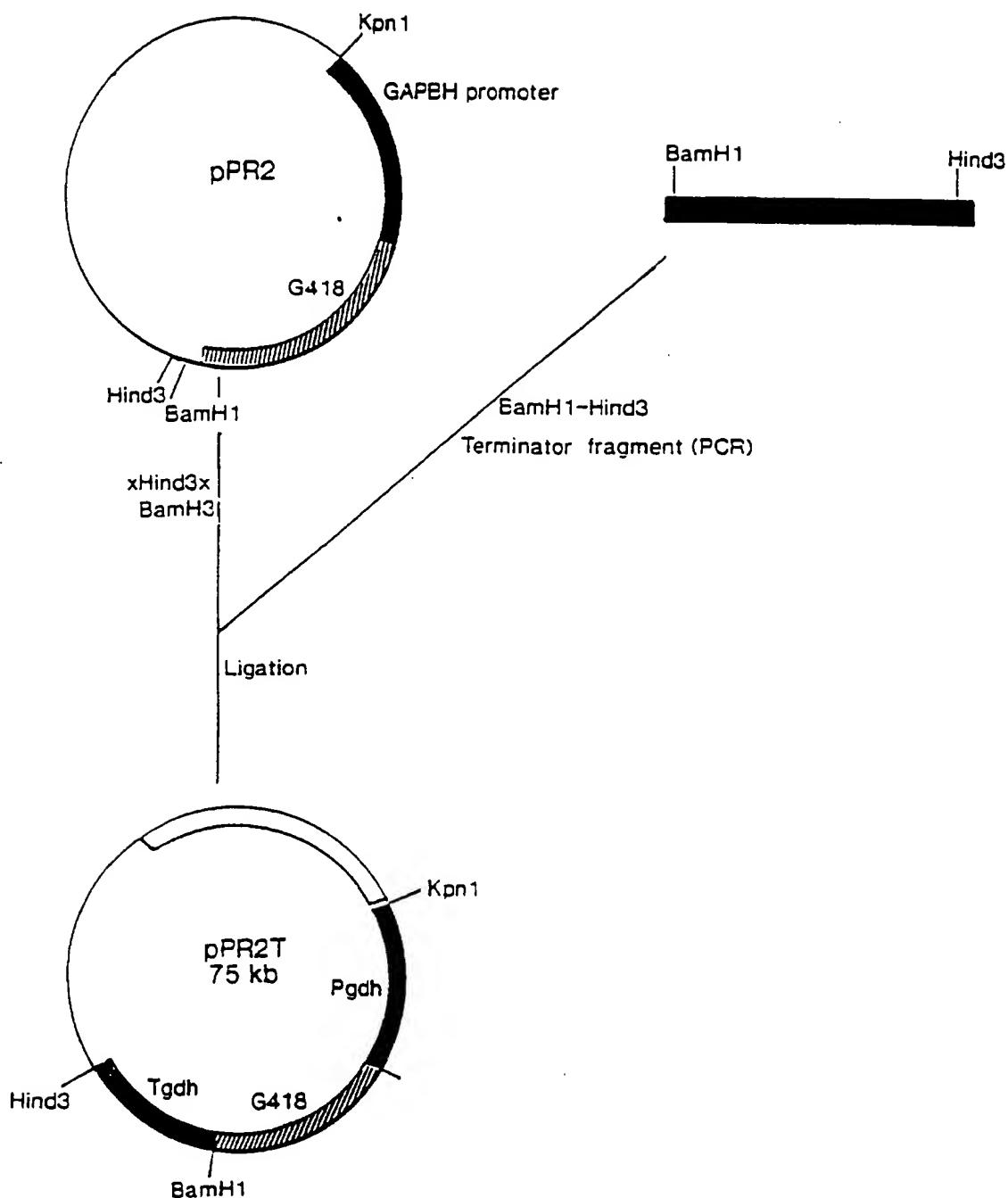


FIG. 4

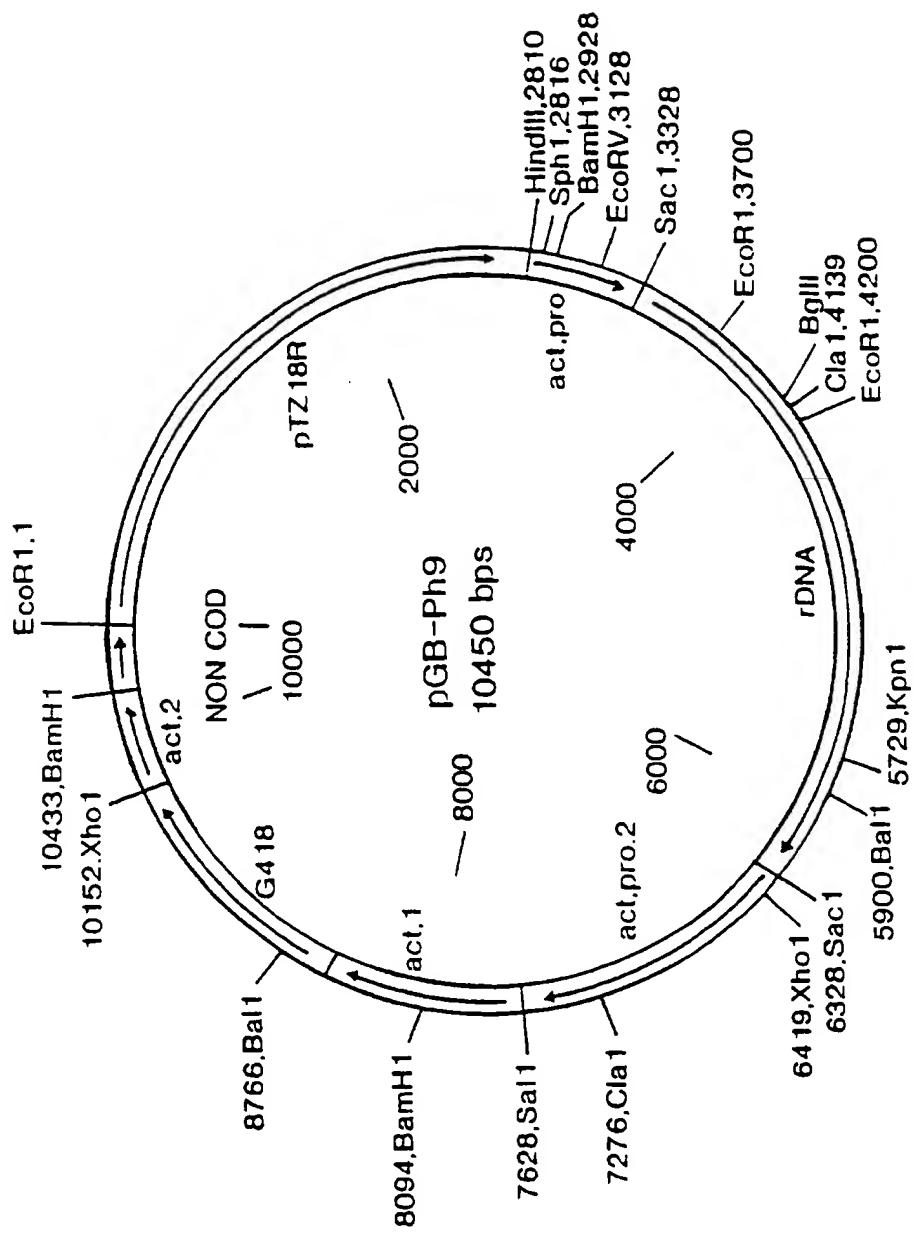
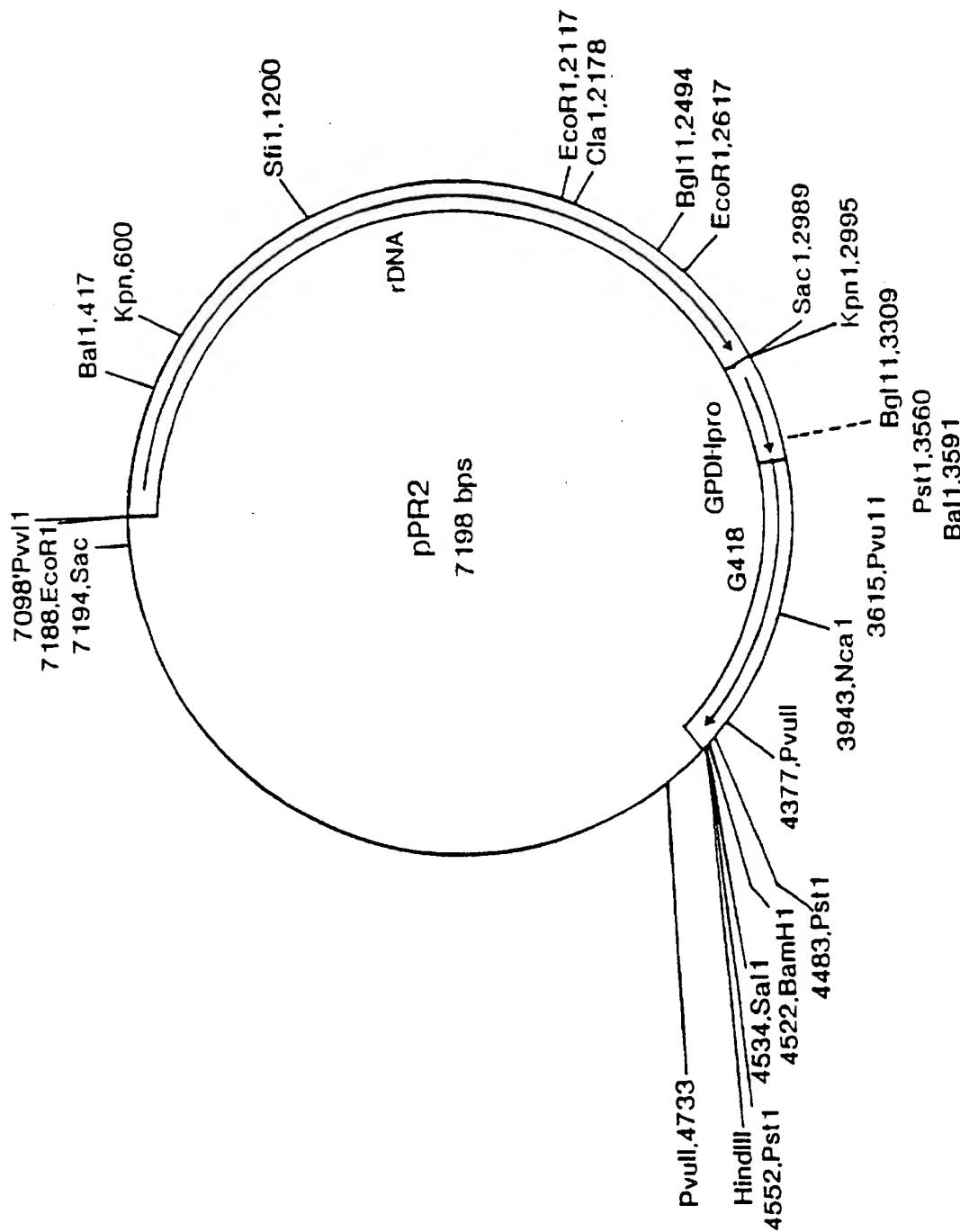


FIG. 5



SUBSTITUTE SHEET (RULE 26)

FIG. 6

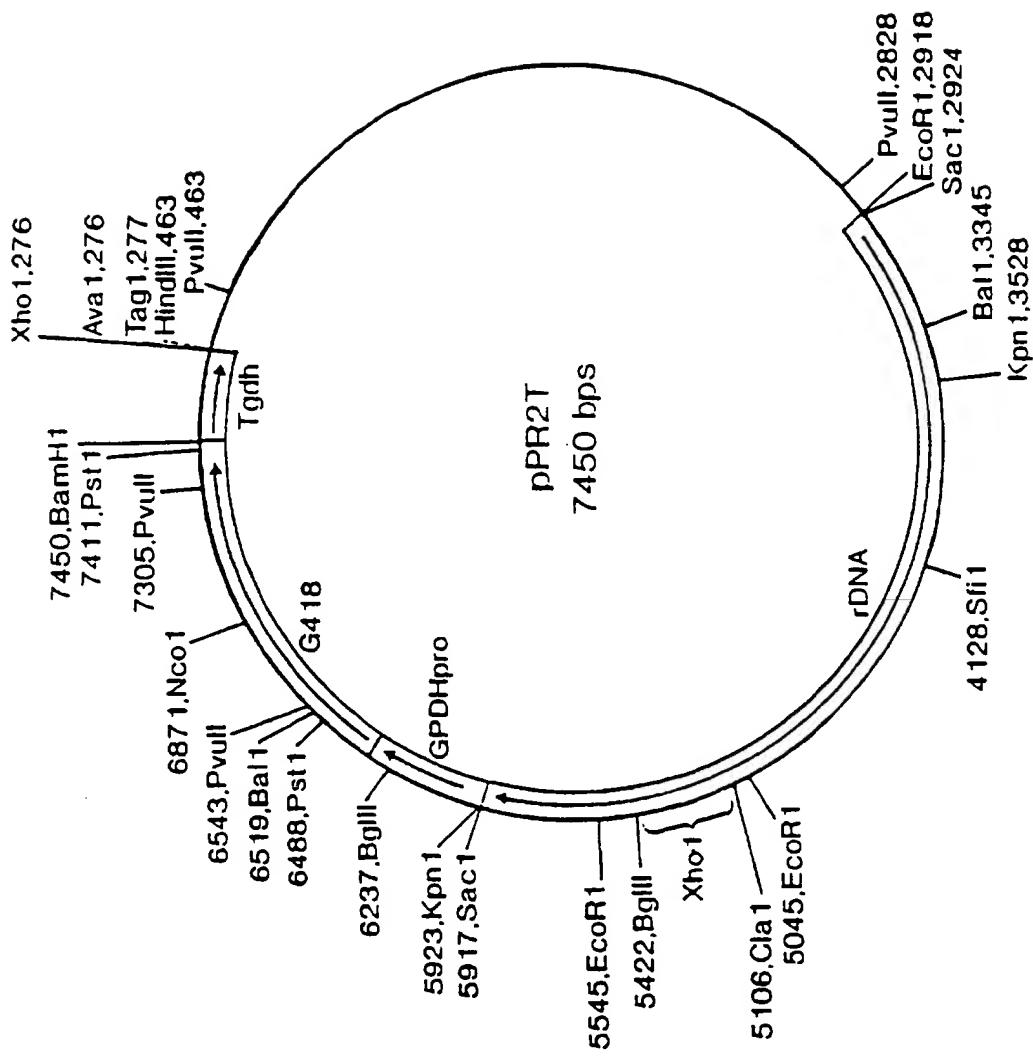


FIG. 7

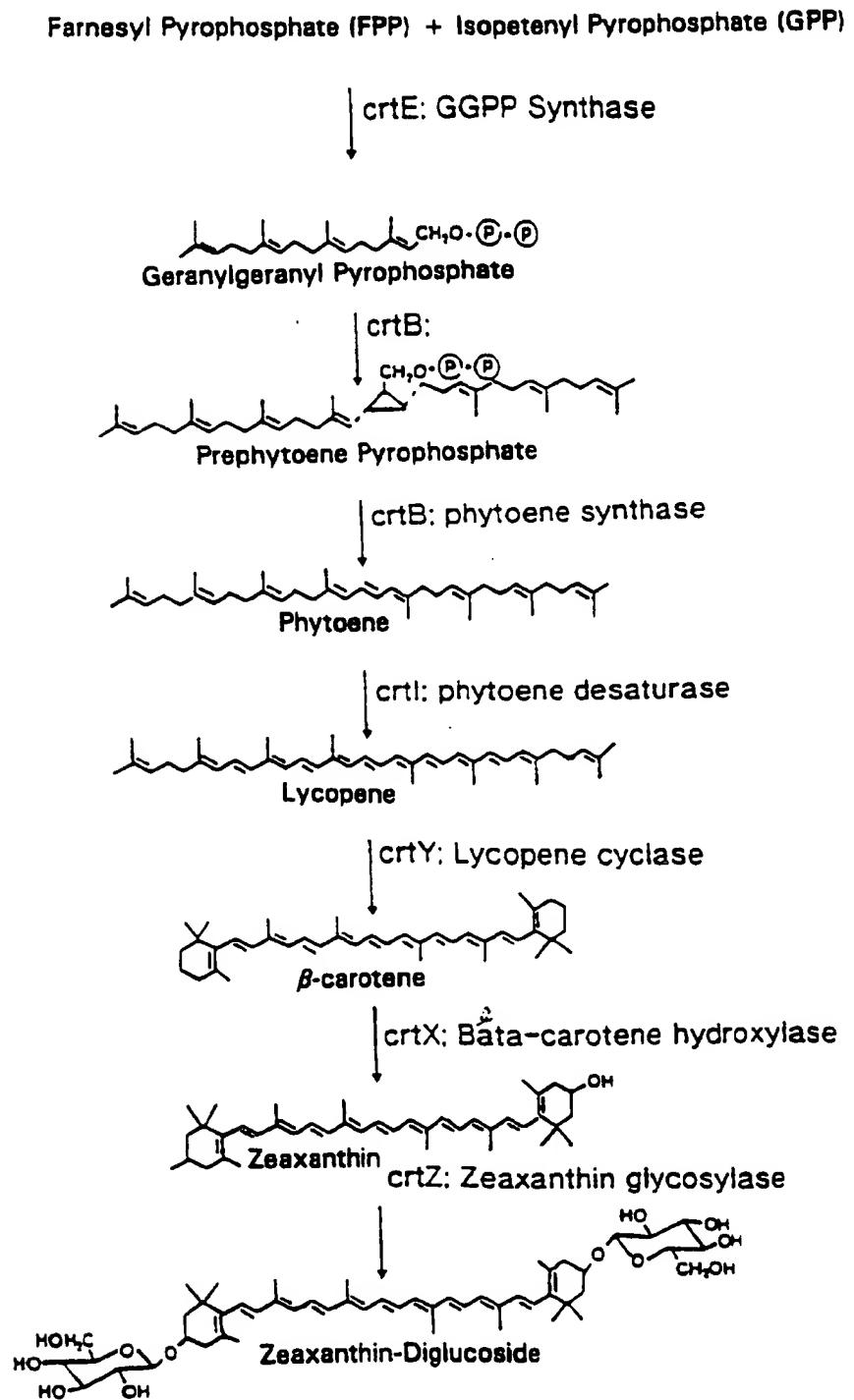
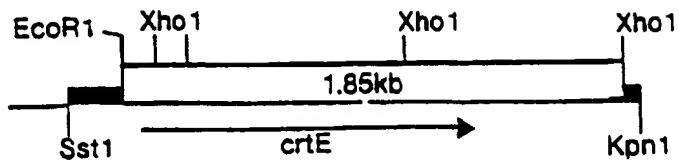
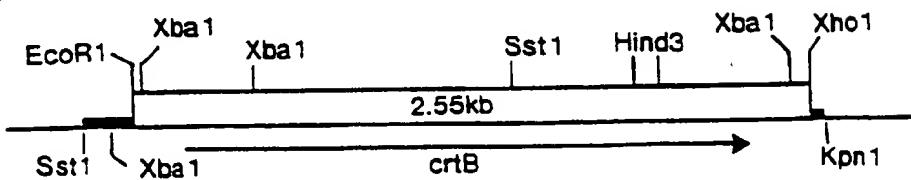
Carotenoid Biosynthetic Pathway of *Erwinia uredovora*

FIG. 8

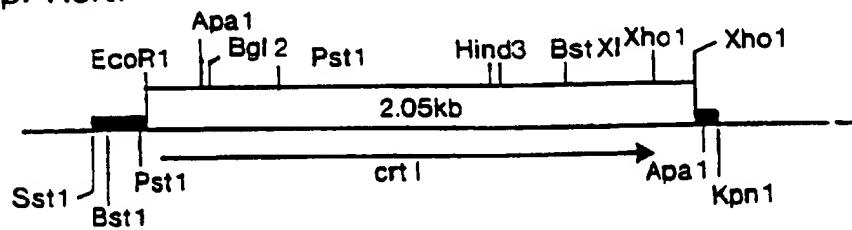
A pPRcrtE



B pPRcrtBY



C pPRcrtI



pPRcrtY

1.0kb

FIG. 9

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 96/05887

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/81 C12N1/16 C07K14/39 C12N9/02 C12N15/53
 C12N15/52 C12N15/60 C12P23/00 C12N1/21 // (C12N1/16,
 C12R1:645), (C12N1/21, C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANALES DE LA REAL ACADEMIA DE FARMACIA, vol. 61, no. 4, 1995, pages 463-471, XP000577134 J. ANDRIO ET AL.: "Transformación de Phaffia rhodozyma utilizando el método del acetato de litio." summary, page 463, page 468, paragraph 3 see page 464, paragraph 1 ---	1,6-8, 12,14, 17-19, 23,25, 27, 33-35, 40,44,45
X	EP 0 590 707 A (GIST BROCADES NV) 6 April 1994 cited in the application	1,6-12, 14, 17-19, 23-25, 27-35, 40-50
Y	see the whole document ---	26,51,52 -/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

Z document member of the same patent family

3

Date of the actual completion of the international search

Date of mailing of the international search report

1.2.96.97

5 June 1997

Name and mailing address of the ISA

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 Fax (+ 31-70) 340-3016

Authorized officer

Hix, R

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 96/05887

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOTECHNOLOGY TECHNIQUES 9 (7). 1995. 509-512. ISSN: 0951-208X, XP000578607 ADRIOL J L ET AL: "Transformation of the astaxanthin-producing yeast Phaffia rhodozyma." cited in the application see the whole document ---	1,6-12, 14, 17-19, 23,25, 27-35, 40-50 26,51,52
Y		
X	MOLECULAR & CELLULAR BIOLOGY, vol. 10, no. 10. October 1990, pages 5064-5070, XP000577173 T.J. SCHIDHAUSER ET AL.: "Cloning sequencing and photoregulation of al-1, a carotenoid biosynthetic gene of Neurospora crassa." see the whole document ---	32-34, 41,42
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 264, no. 22, 5 August 1989, pages 13109-13113, XP000577175 G.E. BARTLEY ET AL.: "Carotenoid biosynthesis in photosynthetic bacteria" see the whole document ---	32,33
X	MOL. GEN. GENET., vol. 216, April 1989, pages 254-268, XP000577174 G.A. ARMSTRONG ET AL.: "Nucleotide sequence, organisation and nature of the protein products of the carotenoid biosynthesis gene cluster of Rhodobacter capsulatus." see the whole document ---	32,33
X	EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 233, no. 1, 1 October 1995, pages 238-248, XP000578408 BOTELLA J A ET AL: "A CLUSTER OF STRUCTURAL AND REGULATORY GENES FOR LIGHT-INDUCED CAROTENOGENESIS IN MYXOCOCCUS XANTHUS" see the whole document ---	32,33
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3		-/-

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/EP 96/05887

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DEVELOPMENTAL BIOLOGY, vol. 170, 1 January 1995, pages 626-635, XP000578443 ARPAIA G ET AL: "LIGHT AND DEVELOPMENT REGULATE THE EXPRESSION OF THE ALBINO-3 GENE IN NEUROSPORA CRASSA" see the whole document ---	32,33
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 16, 22 April 1994, pages 12060-12066, XP000577176 T.J. SCHMIDHAUSER ET AL.: "Characterisation of al-2, the Phytoene Synthase gene of Neurospora crassa." see the whole document ---	32-34
P,X	WO 96 28545 A (KIRIN BREWERY ; KAJIWARA SUSUMU (JP); MISAWA NORIHIKO (JP); KONDO K) 19 September 1996 see the whole document ---	1,6, 9-12,17, 18, 23-25, 28-35, 40-49
T	GENE (AMSTERDAM) 184 (1). 1997. 89-97. ISSN: 0378-1119, XP000646757 WERY J ET AL: "High copy number integration into the ribosomal DNA of the yeast Phaffia rhodozyma." see the whole document ---	1-52
A	DATABASE WPI Section Ch, Week 9331 Derwent Publications Ltd., London, GB; Class D16, AN 93-247564 XP002011179 & JP 05 168 465 A (LION CORP) , 2 July 1993 see abstract ---	1-52
A	J. MICROBIOL. BIOTECHNOL. (1992), 2(1), 46-9 CODEN: JOMBES, 1992. XP000571764 KOH, MOO SUK ET AL: "Construction of astaxanthin overproducing strain of Phaffia rhodozyma by protoplast fusion" see the whole document ---	1-52
		-/-
3		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 96/05887

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. MICROBIOL. BIOTECHNOL. (1995), VOLUME DATE 1995, 5(6), 370-2 CODEN: JOMBES;ISSN: 1017-7825, 1995, XP000571765 CHUN, SOON BAI ET AL: "Cloning of autonomously replicating sequence from Phaffia rhodozyma" see the whole document ---	
A	WO 92 22648 A (VILLADSEN INGRID STAMPE) 23 December 1992 ---	
A	EP 0 474 347 A (QUEST INT) 11 March 1992 ---	
A	FEMS (FED EUR MICROBIOL SOC) MICROBIOL LETT 93 (3). 1992. 221-226. CODEN: FMLED7 ISSN: 0378-1097, XP000569541 CHUN S B ET AL: "STRAIN IMPROVEMENT OF PHAFFIA - RHODOZYMA BY PROTOPLAST FUSION." -----	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 96/05887

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see continuation-sheet

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest:

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 96/05887

FURTHER INFORMATION CONTINUED FR M PCT/ISA/210

1. Recombinant DNA comprising a transcription promoter and downstream region to be expressed where the transcription promoter comprises a region found upstream of a highly expressed **Phaffia** gene, method of transforming a **Phaffia** strain where the transcription promoter is from a **glycolytic pathway gene**, to express a downstream sequence, recombinant DNA thereof, including a selective agent and the transformed **Phaffia** strains : Claims 2, 3, 13, 36 and 37 {completely} and Claims 1, 6 to 14, 17 to 19, 22 to 27, 33 to 35 and 40 to 45 and 51 {partially}.
2. Recombinant DNA comprising a transcription promoter and downstream region to be expressed where the transcription promoter comprises a region found upstream of a highly expressed **Phaffia** gene, method of transforming a **Phaffia** strain where the transcription promoter is from a **ribosomal protein**, to express a downstream sequence, recombinant DNA thereof and the transformed **Phaffia** strains: Claims 4, 5, 15, 16, 38 and 39 {completely} and Claims 1, 6 to 12, 14, 17 to 19, 22 to 27, 33 to 35 and 40 to 45 and 51 {partially}.
3. An isolated DNA fragment comprising a **Phaffia** GAPDH-gene and use in the construction of a DNA construct: Claims 20 to 21 {completely} and Claim 22 {partially}.
4. An isolated DNA sequence coding for an enzyme involved in the carotenoid biosynthetic pathway of **Phaffia rhodozyme** and recombinant DNA comprising a transcription promoter and the downstream region to be expressed codes for an enzyme involved in the **carotenoid biosynthesis pathway** and the transformed **Phaffia** strains comprising said DNA : Claims 1, 6, 9 to 12, 14, 17 to 19, 23 to 27, 28 to 35 and 40 to 50 {partially}
5. An isolated DNA sequence coding for an enzyme involved in the carotenoid biosynthetic pathway of **Phaffia rhodozyme**, and recombinant DNA comprising a transcription promoter and the downstream region to be expressed codes for an enzyme involved in the carotenoid biosynthesis pathway and the transformed **Phaffia** strains comprising said DNA, where the enzyme has **isopentenyl pyrophosphate isomerase activity** : Claims 1, 6, 9 to 12, 14, 17 to 19, 23 to 27, 28 to 35 and 40 to 50 {partially}

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INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 96/05887

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

6. An isolated DNA sequence coding for an enzyme involved in the carotenoid biosynthetic pathway of *Phaffia rhodozyme*, and recombinant DNA comprising a transcription promoter and the downstream region to be expressed codes for an enzyme involved in the carotenoid biosynthesis pathway and the transformed *Phaffia* strains comprising said DNA, where the enzyme has **geranylgeranyl pyrophosphate synthase activity** : Claims 1, 6, 9 to 12, 14, 17 to 19, 23 to 27, 28 to 35 and 40 to 50 {partially}
7. An isolated DNA sequence coding for an enzyme involved in the carotenoid biosynthetic pathway of *Phaffia rhodozyme* and recombinant DNA comprising a transcription promoter and the downstream region to be expressed codes for an enzyme involved in the carotenoid biosynthesis pathway and the transformed *Phaffia* strains comprising said DNA, where the enzyme has **phytoene synthase activity** : Claims 1, 6, 9 to 12, 14, 17 to 19, 23 to 27, 28 to 35 and 40 to 50 {partially}
8. An isolated DNA sequence coding for an enzyme involved in the carotenoid biosynthetic pathway of *Phaffia rhodozyme* and recombinant DNA comprising a transcription promoter and the downstream region to be expressed codes for an enzyme involved in the carotenoid biosynthesis pathway and the transformed *Phaffia* strains comprising said DNA, where the enzyme has **phytoene desaturase activity** : Claims 1, 6, 9 to 12, 14, 17 to 19, 23 to 27, 28 to 35 and 40 to 50 {partially}
9. An isolated DNA sequence coding for an enzyme involved in the carotenoid biosynthetic pathway of *Phaffia rhodozyme* and recombinant DNA comprising a transcription promoter and the downstream region to be expressed codes for an enzyme involved in the carotenoid biosynthesis pathway and the transformed *Phaffia* strains comprising said DNA where the enzyme has **lycopene cyclase activity** : Claims 1, 6, 9 to 12, 14, 17 to 19, 23 to 27, 28 to 35 and 40 to 50 {partially}
10. Method for the isolation of a promoter from a gene expressed in *Phaffia* : Claim 52 {completely}

INTERNATIONAL SEARCH REPORT

Invention on patent family members

International Application No

PCT/EP 96/05887

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP 0590707 A	06-04-94	AU 673847 B AU 4624293 A CA 2105957 A FI 933993 A JP 7501225 T WO 9406918 A NO 933250 A NZ 248628 A		28-11-96 17-03-94 12-03-94 12-03-94 09-02-95 31-03-94 14-03-94 27-02-96
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